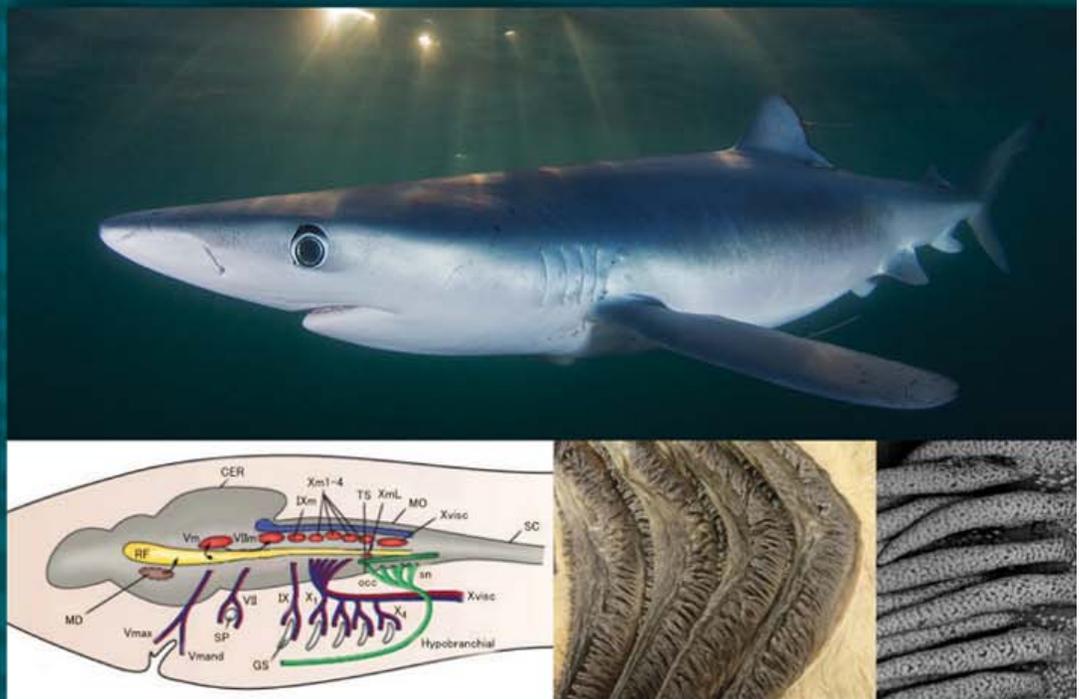


Fish Physiology
Volume 34B

Physiology of Elasmobranch Fishes

Internal Processes



Robert E. Shadwick, Anthony P. Farrell,
and Colin J. Brauner

SERIES EDITORS: Anthony P. Farrell and Colin J. Brauner



PHYSIOLOGY OF
ELASMOBRANCH FISHES:
INTERNAL PROCESSES

This is Volume 34B in the

FISH PHYSIOLOGY series

Edited by Anthony P. Farrell and Colin J. Brauner

Honorary Editors: William S. Hoar and David J. Randall

A complete list of books in this series appears at the end of the volume

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125, London Wall, EC2Y 5AS.
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA
225 Wyman Street, Waltham, MA 02451, USA
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK

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British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress.

ISBN: 978-0-12-801286-4

ISSN: 1546-5098

For Information on all Academic Press publications
visit our website at <http://store.elsevier.com/>

Typeset by MPS Limited, Chennai, India
www.adi-mps.com



Publisher: Janice Audet
Senior Acquisition Editor: Kristi A.S. Gomez
Editorial Project Manager: Pat Gonzalez
Production Project Manager: Lucía Pérez
Designer: Matthew Limbert

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PREFACE

Elasmobranchs consist of sharks, skates and rays which, along with their sister group holocephalans, comprise the extant cartilaginous fishes or class Chondrichthyes. Elasmobranchs hold an important position in the evolutionary history of jawed vertebrates, with fossil evidence of ancestral forms dating back close to 400 million years. There are about 1000 living species of elasmobranchs, mostly marine, all with distinctive features: skeletal elements formed of prismatic calcified cartilage, placoid dermal scales, teeth that are shed and replaced regularly, an osmoregulatory strategy to keep body fluids nearly iso-osmotic with the surrounding water, internal fertilization with many species bearing live young, and highly developed sensory systems including electroreception. Some also elevate their core body temperatures by retaining muscle heat via vascular heat exchangers.

Because of their evolutionary significance and unusual physiology elasmobranchs are of great interest to biologists in general and fish physiologists in particular. This two part volume on the Physiology of Elasmobranch Fishes is modeled on a previous comprehensive treatment of this field published over 25 years ago (*Physiology of Elasmobranchs*, edited by T.J. Shuttleworth in 1989). In that time much has changed in terms of interest and breadth of research on elasmobranchs, and increased public awareness of their importance in oceanic ecosystems and decimation due to commercial fishing pressures. Over the past two decades research efforts and published works on elasmobranch physiology have greatly expanded and now include more sophisticated laboratory work, increased field measurements, a notable increase in the diversity of species studied, and a particular boom in lab and field-based studies of large pelagic sharks. Interestingly, virtually all experimental physiology studies of elasmobranchs have been conducted on wild-caught animals. There are no aquaculture sources for

research specimens, in contrast to the situation for physiologists who work with teleosts, many being supplied from aquaculture stocks that have been subjected to various degrees of genetic manipulation in their domestication. This certainly makes physiological studies of elasmobranchs more difficult, but it is also unusual in the sense that the specimens studied are not influenced by captive breeding artifacts. However, laboratory investigations are still impractical with many elasmobranch species which have relatively large body size, live in remote habitats, and are continuous swimmers.

Here we present a broad exploration of our current knowledge of elasmobranch physiology in a two-part volume. Aspects of elasmobranch structure and interactions with their environment are covered in volume 34A, while volume 34B expands on internal physiological processes. Both parts are fully integrated by cross-referencing between the various chapters, and they share a common index. Twenty-eight authors with appropriate expertise and active research programs in the field have contributed sixteen chapters. Each synthesizes the available data into a comprehensive review of the topic, while also comparing elasmobranchs to other groups of fishes and providing a perspective on future research problems and directions.

Volume 34A begins with a well-illustrated description of the evolutionary history of cartilaginous fishes, highlighting relationships between elasmobranchs, holocephalans, and their ancestors. Chapter 2 follows with a very detailed review of the battery of sensory capabilities found in elasmobranchs, including the more unusual electric and magnetic senses. By examining species from different habitats this chapter investigates how elasmobranchs use their sensory systems to sample their “sensoryscape”, and how this influences their behavioral responses to environmental stimuli. The structure of elasmobranch gills is described and illustrated in striking detail in Chapter 3 where the diversity among elasmobranchs as well as important differences from other fish groups are highlighted. Chapter 4 presents a comprehensive review of the muscular and skeletal anatomy and the mechanics that support a range of feeding behaviors, from simple ancestral biting to more derived suction and filter feeding. Skeletal muscle structure and contractile properties are reviewed in Chapter 5, with an emphasis on how elasmobranch muscle (of which we know comparatively little) compares with muscle in other fishes, and how relatively large body size in sharks confounds such comparisons. In Chapter 6 elasmobranch locomotor diversity, hydrodynamics, and energetics are discussed in comparison with teleosts, and special structures such as the heterocercal tail and the denticled skin are highlighted. Looking to the potential impact of global ocean change, new evidence on temperature effects on locomotor performance is also presented. Chapter 7 presents a comprehensive review of the diverse range of elasmobranch strategies for reproduction, from

oviparity to different forms of viviparity and a survey of modes of embryonic nutrition such as oophagy and intrauterine cannibalism. Finally, Chapter 8 explores the advantages of conducting physiological studies in the field, with descriptions of current technologies and examples of new findings. These innovative approaches are opening new opportunities to study larger species in their natural environment.

Volume 34B begins with two chapters on the function and control of cardio-respiratory systems. Chapter 1 compares elasmobranch and teleost cardiovascular systems, with emphasis on neural, endocrine and paracrine control features. This chapter also considers how various species might experience cardiovascular challenges associated with increased temperature and hypoxia in future oceans. Chapter 2 then describes the physiology of respiratory systems in elasmobranchs, with emphasis on the generation of respiratory rhythm, sensory inputs, motor output patterns, and the integration of control and synchrony of the cardio-respiratory systems. The physiology of oxygen and carbon dioxide transport in elasmobranchs is reviewed in Chapter 3, focusing on functional modifications of hemoglobin and carbonic anhydrase. In addition, adaptations for respiratory gas transport in conditions of exercise, hypoxia, salinity, temperature and in some species, regional heterothermy are reviewed. The next two chapters deal with systems to control ion and fluid balance, highlighting the unusual reliance on urea. Chapter 4 describes how marine elasmobranchs maintain osmotic balance by the use of organic osmolytes (mainly urea and methylamines), making them hypo-ionic osmoconformers, while euryhaline species have reduced osmolytes and function as hyperosmotic regulators. Specific mechanisms used by elasmobranchs to balance acid and base, ions, and nitrogenous wastes by the gill, kidney, gut and rectal gland are detailed in Chapter 5, with specific comparisons among species inhabiting marine, freshwater, and intermediate salinity habitats. Chapter 6 links feeding ecology to physiology in elasmobranchs, and includes a discussion of several analytical techniques used to determine diet composition for different feeding behaviors. The anatomy of the digestive tract is covered in detail, and digestive processes specific to the demands of different ecological niches occupied are reviewed. Chapter 7 reviews metabolic processes in elasmobranchs, clearly demonstrating that their metabolic organization and regulatory mechanisms are very much driven by the need to continuously synthesize and maintain large amounts of urea. This chapter also shows how studies of elasmobranchs can provide broader insight into the evolution of metabolism in the vertebrates. Chapter 8 completes the volume with a detailed and systematic review of endocrine systems in elasmobranchs, emphasizing the importance of hormonal control in many aspects of their physiology. One unusual feature is the production of a corticosteroid unique

to elasmobranchs in the interrenal gland. Although our current knowledge of elasmobranch endocrinology is extensive it has come from studies on but a few of the hundreds of species, suggesting that there is much more to learn in future research, not just in endocrinology, but in all aspects of the physiology of these fascinating fishes.

The editors would like to thank Pat Gonzalez, Kristi Gomez, Lucía Pérez and their production staff at Elsevier for guiding this project from its inception to final publication. We thank the anonymous reviewers who provided constructive comments on the original proposal for this volume, and numerous colleagues who acted as external peer reviewers of the chapter drafts. Finally we thank the team of elasmobranch experts who authored the important written contributions and also aided efforts with their patience and collegial cooperation over the past 2 years.

Robert E. Shadwick
Anthony P. Farrell
Colin J. Brauner

LIST OF ABBREVIATIONS

| | |
|--------------------|---|
| $[Ca^{2+}]_i$ | intracellular (i.e., cytoplasmic) free calcium concentration |
| $[O_2]_{arterial}$ | arterial blood oxygen content |
| $[O_2]_{venous}$ | venous blood oxygen content |
| $[T_{Amm}]$ | plasma total ammonia content |
| ΔH^o | apparent enthalpy of oxygenation |
| ΔP | change in venous pressure |
| ΔP_{O_2} | difference in the partial pressure of oxygen between water and blood (mmHg) |
| ΔV | change in venous blood volume |
| μ | dynamic viscosity of the water |
| α | α -adrenergic receptor |
| β | β -adrenergic receptor |
| β -NHE | β -adrenergic Na^+/H^+ exchanger |
| β END | β endorphin |
| β -LPH | β -lipotropic hormone |
| ΔH^o | enthalpy of heme-oxygenation |
| ΔzH^+ | Haldane coefficient (-4Φ) |
| λ | wavelength |
| λ_{max} | wavelength of maximum absorbance |
| Φ | Bohr coefficient ($\Delta \log P_{50}/\Delta pH$) |
| \dot{Q} | cardiac output |
| 11-KA | 11-ketoandrostenedione |
| 11-KT | 11-ketosterone |
| 1 α -OH-B | 1 α -hydroxycorticosterone |
| 2,3-BPG | 2,3-bisphosphoglycerate |
| 2,3-DPG | 2,3-diphosphoglycerate |
| 5-HT | 5-hydroxytryptamine |
| A | respiratory surface area of the gills (cm ²) |

| | |
|------------------|--|
| AA | arachidonic acid |
| AADC | aromatic L-amino acid decarboxylase |
| ABA | afferent branchial artery |
| ABR | auditory brain stem recording |
| AC | adenylyl cyclase |
| ACE | angiotensin converting enzyme |
| Ach | acetylcholine |
| ACTH | adrenocorticotrophic hormone |
| ADP | adenosine diphosphate |
| AEP | auditory evoked potential |
| A_{lam} | mean bilateral surface area of a gill lamella (mm ²) |
| ALLN | anterior lateral line nerve |
| AMP | adenosine monophosphate |
| Ang II | angiotensin II |
| ANP | atrial natriuretic peptide |
| ARG | arginine |
| ARGase | arginase |
| ASL | argininosuccinate lyase |
| ASS | argininosuccinate synthetase |
| ATP | adenosine triphosphate |
| AVP | arginine vasopressin |
| AVT | arginine vasotocin |
| BADH | betaine aldehyde dehydrogenase |
| BHB | β -hydroxybutyrate |
| BHBDH | β -hydroxybutyrate dehydrogenase |
| BK | bradykinin |
| BL/s | body lengths per second |
| BNP | brain natriuretic peptide |
| CA | carbonic anhydrase |
| cAMP | cyclic adenosine monophosphate |
| CaO ₂ | arterial blood oxygen content |
| CBG | corticosteroid binding globulin |
| CCK | cholecystokinin |
| CCO | cytochrome c oxidase |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| cGMP | cyclic guanosine monophosphate |
| CGRP | calcitonin gene related peptide |
| CICR | calcium induced calcium release |
| Cl ⁻ | chloride ion |
| CNP | C-type natriuretic peptide |
| CNS | central nervous system |
| CO | carbon monoxide |

| | |
|-------------------------------------|--|
| CO ₂ | carbon dioxide |
| CO ₂ | content of O ₂ |
| CoA | coenzyme A |
| COMT | catechol-O-methyltransferase |
| COX | cyclooxygenase |
| CPS | carbamoyl phosphate synthase |
| CPT | carnitine palmitoyl transferase |
| CR | calcitonin receptor |
| CRF | corticotrophic releasing factor |
| CRG | central rhythm generator |
| CRLR | calcitonin receptor-like receptor |
| CRS | cardiorespiratory synchrony |
| CS | citrate synthase |
| CT | calcitonin |
| CvO ₂ | venous blood oxygen content |
| CVPN | cardiac vagal preganglionic neurons |
| DA | dorsal aorta |
| DAG | diacylalkylglycerol=alkyldiacylglycerol |
| DBH | dopamine-β-hydroxylase |
| DC | direct current |
| DHA | docosahexaenoic acid |
| DHT | dihydrotestosterone: |
| DIDS | 4,4-diisothiocyanostilbene-2,2-disulphonic acid (anion exchange inhibitor) |
| DOC | dissolved organic carbon |
| DON | dorsal octavolateralis nucleus |
| DOPA | dihydroxyphenylalanine |
| DVN | dorsal motor nucleus of the vagus nerve |
| E ₂ | 17β-estradiol |
| EC | excitation-contraction |
| ECF | extracellular fluid |
| ECFV | extracellular fluid volume |
| eNOS | endothelial NO synthase |
| EOG | electro-olfactography |
| EROD | ethoxyresorufin-O-deethylase |
| ET _A and ET _B | endothelin receptors subtypes |
| FA | fatty acid |
| FABP | fatty acid binding protein |
| <i>fH</i> | heart rate |
| FSH | follicle stimulating hormone |
| GC | guanylyl cyclase |
| GDH | glutamate dehydrogenase |

| | |
|-------------------------------|--|
| GEP | gastro-entero-pancreatic |
| GFR | glomerular filtration rate |
| GH | growth hormone |
| GHRH | growth hormone releasing hormone |
| GI | gastrointestinal |
| GIT | gastrointestinal tract |
| GLP | glucagon-like peptide |
| GLUT | glucose transporter |
| GnRH | gonadotropin releasing hormone |
| GPC | glycerophosphorylcholine |
| GPI linkage | glycosylphosphatidylinositol linkage |
| GPS | global Positioning System |
| GR | glucocorticoid receptor |
| GRP | gastrin releasing peptide |
| GS | glutamine synthetase |
| GT | gonadotropins |
| GTH | gonadotrophic hormone |
| GTP | guanosine triphosphate |
| GULO | gulono gamma lactone oxidase |
| <i>H</i> | height of a lamella |
| H ⁺ | hydrogen ion (i.e., proton) |
| H ₂ S | hydrogen sulfide |
| Hb | hemoglobin |
| HCl | hydrochloric acid |
| HCO ₃ ⁻ | bicarbonate ion |
| Hct | the percentage of red blood cells in blood |
| HDL | high density lipoprotein |
| HEA | high environmental ammonia |
| HETE | hydroxyeicosatetraenoic acid |
| HIS | hepatosomatic index |
| HKA | H ⁺ , K ⁺ -ATPase |
| HPETE | hydroperoxyeicosatetraenoic acid |
| HPG | hypothalamus–pituitary–gonadal axis |
| HRV | heart rate variability |
| Hz | hertz |
| ICFV | intracellular fluid volume |
| IGF | insulin-like growth factor |
| IHC | immunohistochemistry |
| IHP | inositol hexaphosphate |
| IMP | inosine monophosphate |
| iNOS | inducible nitric oxide synthase |
| IP ₃ | inositol triphosphate |

| | |
|------------------|---|
| IPP | inositol pentaphosphate |
| IQL | inner quadratomandibular ligament |
| IRD | inner ring deiodinase |
| IRI | index of relative importance |
| JGA | juxtaglomerular apparatus |
| <i>K</i> | Krogh coefficient ($\text{mgO}_2 \mu\text{m cm}^{-2} \text{mmHg}^{-1} \text{min}^{-1}$) |
| KKS | kallikrein kinin system |
| K_m | Michaelis constant |
| L-Arg | L-arginine |
| LDH | lactate dehydrogenase |
| LDL | low density lipoprotein |
| L_{fil} | total length of all the gill filaments (cm) |
| LH | luteinizing hormone |
| LT | leukotriene |
| LWS | long wavelength sensitive |
| M | molar |
| MAO | monoamine oxidase |
| MCHC | mean corpuscular hemoglobin concentration |
| MCR | melanocortin receptor |
| MCT | monocarboxylate transporter |
| $\dot{M}O_2$ | O_2 consumption rate |
| MON | medial octavolateralis nucleus |
| MR | mineralocorticoid receptor |
| MRC | mitochondrion-rich cell |
| mRNA | messenger RNA |
| MSH | melanocyte stimulating hormone |
| MWS | medium wavelength sensitive |
| <i>N</i> | newtons |
| N/O ₂ | nitrogen quotient |
| Na ⁺ | sodium ion |
| NAD ⁺ | nicotinamide adenine dinucleotide (oxidized form) |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| NADP | nicotinamide adenine dinucleotide phosphate (oxidized form) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced form) |
| NAG | N-acetyl glutamate |
| NANC | nonadrenergic noncholinergic |
| NCX | Na ⁺ -Ca ²⁺ exchanger |
| NEC | neuroepithelial cell |
| NEFA | nonesterified fatty acid |
| n_H | Hill's cooperativity coefficient at 50% Hb-O ₂ saturation |

| | |
|--------------------|---|
| NHE | Na ⁺ /H ⁺ exchanger |
| NIL | neurointermediate lobe |
| NKA | Na ⁺ , K ⁺ -ATPase |
| NKCC | Na/K/2Cl cotransporter |
| n_{lam} | number of lamellae per unit length on one side of a filament (=lamellar frequency) (mm^{-1}) |
| nNOS | brain or neuronal NOS |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| NOX | NAD(P)H oxidases |
| NPR | natriuretic peptide receptor |
| NPs | natriuretic peptides |
| NPY | neuropeptide Y |
| NTP | nucleoside triphosphates |
| nV | nanovolt |
| O ₂ | oxygen |
| OBs | olfactory bulbs |
| OFT | optimal foraging theory |
| OMZ | oxygen minimum zone |
| ORD | outer ring deiodinase |
| ORNs | olfactory receptor neurons |
| Osm | osmolarity |
| Osm/kg | osmolality |
| OTC | ornithine transcarbamoylase |
| OUC | ornithine urea cycle |
| P4 | progesterone |
| P_{50} | partial pressure at which Hb is 50% saturated |
| PACAP | pituitary adenylate cyclase activating peptide |
| P_{aCO_2} | partial pressure of CO ₂ in arterial blood |
| P_{aO_2} | partial pressure of O ₂ in arterial blood |
| PC | pyruvate carboxylase |
| PC | proconvertase |
| P_{CO_2} | partial pressure of CO ₂ |
| P_{crit} | critical oxygen level |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PFK | phosphofructokinase |
| pGC | guanylate cyclase-linked receptors |
| PGI ₂ | prostacyclin |
| Pgs | prostaglandin |
| pH _a | arterial blood pH |
| pHe | extracellular pH (i.e., blood plasma pH) |
| pHi | intracellular pH |

| | |
|----------|--|
| PI | pars intermedia |
| PiO_2 | inspired oxygen partial pressure |
| PI-PLC | phosphatidylinositol phospholipase-C |
| PK | pyruvate kinase |
| PL | placental lactogen |
| PL-A | phospholipase A |
| PLB | phospholamban |
| PLLN | posterior lateral line nerve |
| PML | palatoquadrate-mandibular connective tissue sheath |
| PMV | partial molal volume |
| PNMT | phenylethanolamine-N-methyl transferase |
| PO_2 | partial pressure of O_2 |
| POMC | pro-opiomelanocortin |
| PP | pancreatic polypeptide |
| PPC | pericardioperitoneal |
| PPD | proximal pars distalis |
| PPi | pyrophosphate |
| PRL | prolactin |
| PTH | parathyroid hormone |
| PTHrP | parathyroid hormone related protein |
| pTRG | para-trigeminal respiratory group |
| PTS | proximal tubular segment |
| PUFA | polyunsaturated fatty acids |
| PvO_2 | partial pressure of O_2 in venous blood |
| PwO_2 | partial pressure of O_2 in water |
| PYY | peptide YY |
| Q_{10} | relative change for a 10° C increase in temperature (temperature coefficient) |
| QFASA | quantitative fatty acid signature analysis |
| <i>R</i> | resistance of the gills |
| RAS | renin angiotensin system |
| RBCs | red blood cells |
| RGC | retinal ganglion cell |
| Rh | rhesus |
| RH | medium wavelength sensitive visual pigment |
| Rhp2 | rhesus protein 2 |
| RM | red muscle |
| ROS | reactive oxygen species |
| RPD | rostral pars distalis |
| RRG | respiratory rhythm generation |
| RVD | regulatory volume decrease |
| RVI | regulatory volume increase |

| | |
|--------------------------|--|
| RVMN | respiratory vagal motor neurons |
| RYR | ryanodine receptor |
| sAC | soluble adenylyl cyclase |
| SCA | stomach content analysis |
| SDA | specific dynamic action |
| SERCA | sarco/endoplasmic reticulum Ca ²⁺ -ATPase |
| sGC | soluble guanylyl cyclase |
| SGLT | sodium-linked glucose transporter |
| ShUT | shark urea transporter |
| SIA | stable isotope analysis |
| SL | somatolactin |
| SNGFR | single nephron glomerular filtration rate |
| SNP | sodium nitroprusside |
| spl | splanchnic nerve |
| SR | sarcoplasmic reticulum |
| SR-RR | sarcoplasmic reticulum ryanodine receptor |
| SST | somatostatin |
| StAR | steroidogenic acute regulatory protein |
| strut | stingray urea transporter |
| SV | spiral valve |
| SWS | short wavelength sensitive |
| T | testosterone |
| <i>t</i> | diffusion distance (μm) |
| T3 | triiodothyronine |
| T ₃ | triiodo-L-thyronine |
| T4 | thyroxine |
| T ₄ | tetraiodothyronine |
| TA | titratable acidity |
| TAG | triacylglycerol |
| TBX | thromboxane |
| TEP | transepithelial potential |
| TG | thyroglobulin |
| TH | tyrosine hydroxylase |
| THs | thyroid hormones |
| TMA | trimethylamine |
| TMAO | trimethylamine-N-oxide |
| TMAOX | trimethylamine-N-oxide oxidase |
| TR | thyroid receptor |
| TRH | thyroid releasing hormone |
| TSH | thyroid stimulating hormone |
| UBB | ultimobranchial bodies |
| <i>U</i> _{crit} | critical or maximal prolonged swimming velocity |

| | |
|------------|---------------------------------------|
| UFR | urine flow rate |
| UI | urotensin I |
| UII | urotensin II |
| UT | urea transporter |
| UV | ultraviolet |
| VHA | V-type H ⁺ -ATPase |
| VIP | vasoactive intestinal (poly)peptide |
| VLDL | very low density lipoprotein |
| V_{\max} | maximal velocity of muscle shortening |
| VNP | ventricular natriuretic peptide |
| VPD | ventral pars distalis |
| Vtg | vitellogenin |
| W | the width or thickness of a lamella |
| wrc | white rami communicans |

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ELASMOBRANCH CARDIOVASCULAR SYSTEM

RICHARD W. BRILL

N. CHIN LAI

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The functional characteristics of elasmobranch and teleost cardiovascular systems are similar at routine metabolic rates. Differences do become apparent, however, in cardiovascular function of high-energy-demand species (e.g., mako shark and yellowfin or skipjack tunas) at maximum metabolic rates. Elasmobranchs have an autonomic nervous system

separable into parasympathetic and sympathetic components. The vagus nerve has a major role in controlling heart rate, although sympathetic innervation of the heart and blood vessels is absent. Elasmobranchs increase cardiac output primarily by increasing stroke volume which, in turn, is primarily determined by ventricular end diastolic volume. End diastolic volume is determined by filling time and venous filling pressure; with the latter being effected by venous tone and venous capacitance. Blood volume and pressure in elasmobranchs are controlled by endocrine (e.g., renin-angiotensin, kallikrein-kinin, and natriuretic peptides) and paracrine (e.g., endothelins, prostaglandins, the gasotransmitters NO and H₂S,) mechanisms. Excitation-contraction (EC) coupling in elasmobranch hearts largely fits the accepted model for vertebrates, although the rise in cytoplasmic calcium is primarily from trans-sarcolemmal sources, which includes Na⁺-Ca²⁺ exchanger (NCX).

Some elasmobranchs populations are severely depleted due to the intersection of life history characteristics, unsustainable rates of fisheries-associated mortality, and environmental degradation. To address these issues effectively will require a better understanding of the elasmobranch cardiovascular physiology – including the ability of various species to withstand the physiological consequences of the increasing temperature and expanding hypoxic zones accompanying global climate change, and the severe acidosis and the plasma ionic imbalances resulting from interactions with fishing gear.

1. INTRODUCTION

In this chapter we review recent advances in our understanding of cardiovascular physiology of elasmobranchs, but we specifically forego an extensive narrative on basic cardiovascular anatomy as detailed descriptions are available in earlier reviews (e.g., [Butler and Metcalfe, 1988](#); [Muñoz-Chápuli, 1999](#); [Satchell, 1999](#); [Tota, 1999](#)). Rather this chapter centers on factors and mechanism affecting and controlling cardiovascular function, as well as the relationship of cardiovascular function to energetics, life style, and habitat. We also briefly review recent work that is applicable to elasmobranch conservation; specifically the relationship of cardiovascular function to the ability of various species to tolerate hypoxia and the effects of directional climate change, and to survive following capture and release from fishing gear.

The phylogenies of the cartilaginous fishes (class Chondrichthyes), and the subclasses Elasmobranchii (sharks and rays) and Holocephali (chimeras), are explained in detail by [Klimley \(2013\)](#) and [Janvier and Pradel, \(2015\)](#). In brief, the Elasmobranchii is separated into subgroups Selachii (sharks) and Batoidea (skates and rays), which show extraordinarily different body morphologies (e.g., the latter are dorso-ventrally flattened, have ventral gill slit openings, and pectoral fins fused to the side of the head

forming wings). The modern sharks, in turn, are considered to have derived from two separate lineages: the squalomorphs (superorder Squalomorphi) and galeomorphs (superorder Galeomorphi), with the former considered the more primitive (Klimley, 2013). Since their major diversification in the Permian ($\sim 250 \times 10^6$ years ago), members of the Elasmobranchii have come to occupy almost all aquatic environments – from the surf zone of the continental shelves, to the brightly lighted surface water of the pelagic zone far from the continents, to abyssal depths (> 3000 m), and even into freshwaters – and to occupy latitudes from the tropics to high Arctic (Klimley, 2013). Elasmobranchs also show a great diversity in life styles, life-histories, feeding strategies, energetics, etc. (Compagno, 1990). Given this great heterogeneity in gross body morphologies and ecologies, we contend that the cardiovascular systems of the ~ 900 – 1000 extant species of cartilaginous fishes undoubtedly have a significant range in functional characteristics. Unfortunately, the functional properties of only a few elasmobranch species have been investigated, and these have largely been small, inshore, demersal species (e.g., catsharks, *Scyliorhinus stellaris* and *S. canicula*; epaulette shark, *Hemiscyllium ocellatum*; Port Jackson shark, *Heterodontus portusjacksoni*; eastern shovelnose ray, *Aptychotrema rostrata*; and spiny dogfish, *Squalus acanthias* and *S. suckleyi*). This situation occurs, in large measure, because studies on cardiovascular function generally require access to live specimens held in captivity, and species which are small enough to be easily and safely instrumented. As a result, our summary of cardiovascular function in elasmobranchs will largely rely on data from a relatively small number of generally inshore species that are primarily from temperate, and more occasionally from tropical, areas. We readily admit, therefore, that many of our conclusions may not be universally true for all extant elasmobranch species, but rather be applicable primarily to species sharing a recent common phylogeny, or those belonging to one of the ecomorphotype categories to which they can be assigned (Compagno, 1990).

2. CARDIOVASCULAR FUNCTION AND ENERGETICS

The rate at which the cardiovascular system delivers oxygen to the tissues is described by the Fick equation (Schmidt-Nielsen, 1979)

$$[\text{O}_2]_{\text{delivery}} = \text{SV} * \text{HR} * ([\text{O}_2]_{\text{arterial}} - [\text{O}_2]_{\text{venous}}) \quad (1.1)$$

where

$[\text{O}_2]_{\text{delivery}}$ = rate of O_2 delivery by the cardiovascular system (e.g., $\text{mg O}_2 \text{ min}^{-1} \text{ kg}^{-1}$)

SV = stroke volume (e.g., $\text{ml beat}^{-1} \text{ kg}^{-1}$)

HR = heart rate (e.g., beats min^{-1})

$[\text{O}_2]_{\text{arterial}}$ = arterial blood oxygen content (e.g., $\text{mg O}_2 \text{ ml}^{-1}$)

$[\text{O}_2]_{\text{venous}}$ = venous blood oxygen content (e.g., $\text{mg O}_2 \text{ ml}^{-1}$).

Each of these parameters show changes over evolutionary time (i.e., between species), but also within an individual over the seconds or minutes required to go from resting or routine to maximum aerobic metabolic rates. We therefore divide this topic into two parts and discuss these two situations separately. We begin by comparing cardiovascular function at routine activity levels across representative elasmobranch and teleost species, and then do the same when examining changes occurring with increases metabolic rate. Because the metabolic rates of various elasmobranch species have been comprehensively reviewed (Brett and Blackburn, 1978; Lowe and Goldman, 2001; Carlson et al., 2004; Bernal et al., 2012), we do not repeat an extensive discussion of those data. We do note, however, that we are ignoring the possible contribution of cutaneous oxygen uptake and direct oxygen utilization by the gills, both of which can result an overestimation of cardiac output based on $[\text{O}_2]_{\text{arterial}}$ and $[\text{O}_2]_{\text{venous}}$ and simultaneously measured metabolic rate using the Fick equation, at least in teleosts (Thorarensen et al., 1996; Farrell et al., 2014). To the best of our knowledge, this has never been investigated in elasmobranchs and it is possible that the concern may be irrelevant because of the dermal denticles and thickened and nonvascular skin of elasmobranchs limit cutaneous gas exchange.

It's long been recognized that the functional properties of the cardiovascular system in teleosts (i.e., those shown in Eq. 1.1 that determine rates of oxygen delivery to the tissues) are necessarily correlated with species' energetics (i.e., routine and maximum aerobic metabolic rates) (e.g., Brill, 1996; Brill and Bushnell, 1991, 2001; Farrell, 1991, 1996; Bernal et al., 2012). Although, as extensively discussed by Coulson (1977, 1986, 1997), in evolutionary terms the specific cause-and-effect relationships between cardiovascular function and metabolic rate are complex. And it is still an open question as to whether high performance cardiovascular systems are required for high metabolic rates, or conversely that high metabolic rates are the result of high rates of oxygen and metabolic substrates delivered by the cardiovascular system to the tissues; although the latter is the explanation we favor. Nonetheless, in the following paragraphs we examine each of the parameters in Eq. 1.1 in relation to routine metabolic rate using species-specific examples. Functional gill morphology (e.g., blood flow pathways, gill surface area, diffusion barrier thickness) is also intimately connected with metabolic rate (Wegner et al., 2010), but this topic is reviewed by Wegner (2015) and will not be discussed further here.

2.1. Oxygen Transport by the Cardiovascular System

We start by comparing cardiovascular function in the inshore temperate demersal catsharks (*Scyliorhinus* spp.), the inshore tropical demersal epaulette shark and eastern shovelnose ray, and the thermoconserving pelagic shortfin mako shark (*Isurus oxyrinchus*). These species have metabolic rates (Table 1.1) in the approximate middle and at the upper end of the range reported for elasmobranchs, respectively (Lowe and Goldman, 2001; Carlson et al., 2004; Bernal et al., 2012). The catsharks, epaulette shark, and eastern shovelnose ray have ventricular masses and SV approximately half those of the mako shark (Table 1.1). As a result, former have lower cardiac output ($=SV*HR$) than mako shark, even though the species have roughly equivalent HR at their routine activity levels (Table 1.1) when corrected for differences in temperature by assuming heart rate doubles for every 10°C increase in temperature (i.e., has a $Q_{10} \approx 2$). Catsharks, epaulette shark, and eastern shovelnose ray also have lower $[O_2]_{\text{arterial}}$ compared to mako shark (Table 1.1) reflecting their lower hematocrit. Therefore, in terms of cardiovascular function described by Eq. 1.1, it is these two characteristics (i.e., elevated SV and $[O_2]_{\text{arterial}}$) that appear to be the primary reasons that the routine metabolic rates of catsharks are approximately one tenth of those of mako shark (Table 1.1). Other authors have reached similar conclusions when comparing the hematology (a surrogate for $[O_2]_{\text{arterial}}$) and heart masses (a surrogate for SV) across a range of shark and ray species (Poupa and Ostadal, 1969; Emery et al., 1985; Emery, 1986; Baldwin and Wells, 1990; Filho et al., 1992; Grim et al., 2012); although in most instances there were no corroborating data on *in vivo* cardiovascular function or metabolic rates for the species studied. Rather differences in the metabolic rates were inferred from species-specific activity patterns or the presence of regional endothermy. The latter has been shown to be correlated with elevated metabolic rates in a range of teleost and elasmobranch species (e.g., Korsmeyer and Dewar, 2001; Bernal et al., 2012).

The correlation of a large heart mass (and therefore presumably large SV) and high rates of $[O_2]_{\text{delivery}}$ (i.e., aerobic metabolic rates) becomes less clear when comparisons are made across a group of more active coastal and pelagic shark species (Emery, 1985; Emery et al., 1985). Both mako and great white sharks (*Carcharodon carcharias*) are regional endotherms and both appear to have elevated metabolic rates compared to other elasmobranch species (Bernal et al., 2012). Yet only the latter has a clearly larger heart mass, and therefore a presumably larger SV (Fig. 1.1). Although no data on SV are available for lemon (*Negaprion brevirostris*) or sandbar (*Carcharhinus plumbeus*) sharks, given the lack of differences in heart mass between these two species and shortfin mako shark (Fig. 1.1), we argue it is

Table 1.1

Cardiorespiratory parameters in representative elasmobranchs (catsharks, *Scyliorhinus* spp.; epaulette shark, *Hemiscyllium ocellatum*; shovelnose ray, *Aptychotrema rostrata*; and shortfin mako shark, *Isurus oxyrinchus*) and teleost species (rainbow trout, *Oncorhynchus mykiss*; skipjack tuna, *Katsuwonus pelamis*; and yellowfin tuna, *Thunnus albacares*)

| | Catsharks ^a | Epaulette shark ^b | Shovelnose ray ^b | Rainbow trout ^c | Mako Shark ^d | Yellowfin tuna ^c | Skipjack tuna ^c |
|--|------------------------|------------------------------|-----------------------------|----------------------------|-------------------------|-----------------------------|----------------------------|
| Temperature (°C) | 15–19 | 28 | 28 | 10 | 20–22 | 25 | 25 |
| Speed (body lengths s ⁻¹) | | | | | 0.45 | 1.0–1.3 | 1.6–2.2 |
| Activity level | rest | rest | rest | rest | routine | routine | routine |
| Mass (kg) | ~0.9 | 1.29±0.04 | 1.54±0.06 | 0.9–1.5 | 5–12 | ~1–2 | ~1–2 |
| Metabolic rate (mg O ₂ kg ⁻¹ h ⁻¹) | 37 | ~95 | ~73 | 48 | 344–369 ^e | 776 | 974 |
| Heart rate (beats min ⁻¹) | 35 | ~60 | ~56 | 38 | 28–78 | 62–97 | 79 |
| Cardiac output (ml min ⁻¹ kg ⁻¹) | 44 | ~44 | ~39 | 18 | 32–70 | 115 | 132 |
| Stroke volume (ml kg ⁻¹) | 1.2 | ~0.72 | ~0.72 | 0.46 | 1.6–3.0 | 1.1 | 1.3 |
| Ventricle mass (% body mass) | 0.08 ^f | 0.05 | 0.07 | 0.08–0.13 ^a | 0.17 ^f | 0.29 | 0.39 |
| Hematocrit (%) | 18 | 13.4±0.7 | 12.2±0.7 | 23 | 20–31 | 27–35 | 34–38 |
| Arterial blood O ₂ content (mg dl ⁻¹) | 5.2 | ~3.2 | ~3.2 | 15 | 7.5–10.7 | 18 | 20 |
| Mean ventral aortic pressure (kPa) | 5.3 | ~3.9 | ~3.9 | 5.2 | 6.5 | 11–12 | 12 |
| Cardiac power (mW g ⁻¹ heart mass) ^f | ~2 ^g | ~5.8 | ~3.8 | 1.2–1.9 | ~4 | ~7–8 | ~7 |

^aData are from Tables 9.1 and 9.2 in [Satchell \(1999\)](#). The reported metabolic rate data agree with those for the same species reported by [Sims \(1996\)](#).

^bData are from [Speers-Roesch et al. \(2012a, b\)](#).

^cData are from [Table 1.1 in Brill and Bushnell \(2001\)](#).

^dData are from [Lai et al. \(1997\)](#) except as noted, all results are from swimming fish with the exception of cardiac output and stroke volume which were measured in anesthetized individuals.

^eMetabolic rate data for shortfin mako sharks are from [Sepulveda et al. \(2007\)](#).

^fData on relative ventricle mass are from [Emery et al. \(1985\)](#), [Davie and Farrell \(1991\)](#), and [Bernal et al. \(2003\)](#).

^gData are from [Driedzic \(1992\)](#).

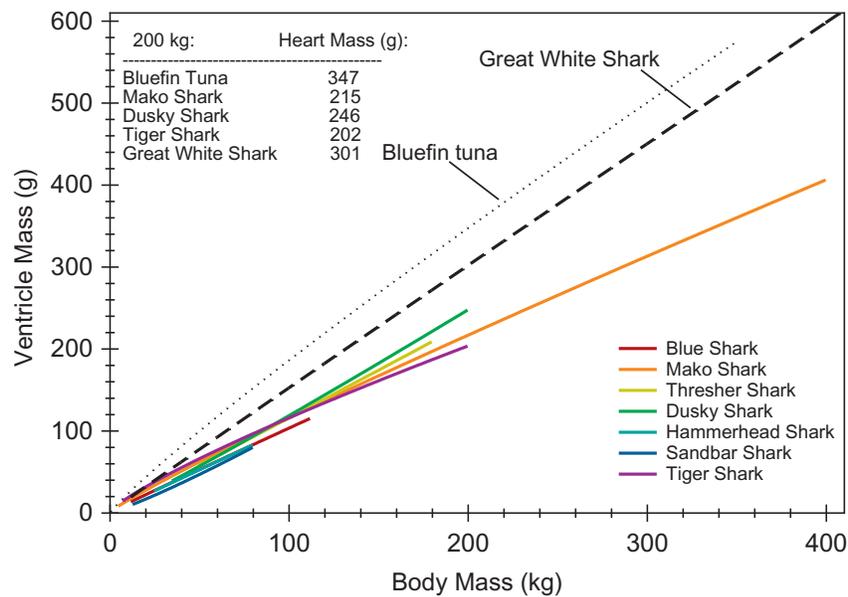


Figure 1.1. Relationship between heart mass (g) and body mass (kg) for various shark species taken from regression equations in [Emery et al. \(1985\)](#). The lengths of the lines show the range of body masses over which data were obtained. The estimated heart mass at a common body mass (200 kg) are also shown. A regression line showing heart mass in a regionally endothermic teleost, bluefin tuna (*Thunnus thynnus*), have been added for comparison. Data from [Poupa et al. \(1981\)](#).

likely that SV is equivalent in all three species. Given their equivalent HR, the higher metabolic rate (i.e., $[O_2]_{\text{delivery}}$) of the mako shark (which is double those of lemon or sandbar sharks at equivalent water temperatures and activity levels, [Table 1.2](#)) cannot be explained by differences in cardiac output (i.e., $HR \times SV$, [Eq. 1.1](#)). Rather the difference appears to be solely due to difference in $[O_2]_{\text{arterial}}$, which is reflected in the shortfin mako shark's ~50–100% higher hematocrit ([Table 1.2](#)).

The matching of the functional properties of the cardiovascular system to species' energetics is also demonstrated when comparisons are made between spotted catsharks and a representative teleost (rainbow trout, *Oncorhynchus mykiss*). These two species have essentially equal routine metabolic rates and equivalent HR, SV, and $[O_2]_{\text{arterial}}$ ([Table 1.1](#)) in spite of the anatomical differences in teleost and elasmobranch respiratory and cardiovascular systems ([Satchell, 1999](#)). Indeed we suggest, as have others (e.g., [Satchell, 1999](#)), that at least in species whose routine metabolic rates are at the lower to middle end of the range of those exhibited by elasmobranchs and teleosts ([Bernal et al., 2012](#); [Brill and Bushnell, 1991, 2001](#)), the differences in the functional characteristics of their cardiovascular systems are relatively small ([Table 1.1](#)) in spite of the very different evolutionary histories of the two groups. Differences in cardiovascular function at routine metabolic rates

Table 1.2

Metabolic rates and cardiovascular parameters in lemon shark (*Negaprion brevirostris*) and sandbar shark (*Carcharhinus plumbeus*), both of which are active coastal species, and the shortfin mako shark (*Isurus oxyrinchus*), a high-energy-demand pelagic species

| | Swimming speed (BL/s ⁻¹) | O ₂ consumption (mg kg ⁻¹ h ⁻¹) | Heart rate (beats min ⁻¹) | Hematocrit (%) | Arterial blood O ₂ content (mg dl ⁻¹) |
|---|--------------------------------------|---|---------------------------------------|----------------|--|
| ^a Lemon shark (11.1–1.6 kg, 22–25°C) | 0.45 | 240 | 51–56 | 15 | 4.1 |
| ^b Sandbar shark (3 kg, 24–25°C) | 0.47 | 155 | 54 | 17.4 | 5.3 |
| ^c Mako shark (5–12 kg, 20–22°C) | 0.45 | 344–369 | 52 | 20–31 | 7.5–10.7 |

^aData from [Bushnell et al., 1982](#); and [Scharold and Gruber, 1991](#).

^bData from [Dowd et al., 2006](#); [Brill et al., 2008](#); and R.W. Brill and P.G. Bushnell, unpublished observations.

^cData are from [Lai et al., 1997, 2004](#).

between elasmobranchs and teleosts do become apparent, however, when comparisons are made between pelagic, sympatric, and thermoconserving high-energy-demand elasmobranch and teleost fishes: the shortfin mako shark and yellowfin and skipjack tunas (*Thunnus albacares* and *Katsuwonus pelamis*, respectively) ([Table 1.1](#)). Note that the mass specific metabolic rate of shortfin mako shark at routine swimming speeds is approximately half that of the two tuna species, and that this is reflected in the lower [O₂]_{arterial}, HR, and cardiac output of the former ([Table 1.1](#)). These differences are apparent even though there has been a remarkable degree of convergent evolution in lamnid sharks (i.e., members of the order Lamniformes) and tunas in other aspects of their biology – ranging from the biochemical to the gross anatomical level (described in detail by [Bernal et al., 2001, 2003a,b, 2009](#); [Patterson et al., 2011](#)).

With respect to the changes in cardiovascular function accompanying increases in metabolic rate, it is obvious from [Eq. 1.1](#) that increases in [O₂]_{delivery} may be achieved by increases in SV, HR, ([O₂]_{arterial}–[O₂]_{venous}) individually, or any combination thereof. Although available data are limited, at least when making comparisons between elasmobranchs and a representative teleost (rainbow trout), there is only one particular characteristics of their cardiovascular systems distinguishing elasmobranchs from teleosts at high rates of [O₂]_{delivery}. In early work on rainbow trout, increases in cardiac output (i.e., SV*HR) accompanying increases in metabolic rate (i.e., increases [O₂]_{delivery}) due to exercise were shown to be accomplished predominately by increases in SV, with only minor increases in HR ([Fig. 1.2](#)) (e.g., [Kiceniuk and](#)

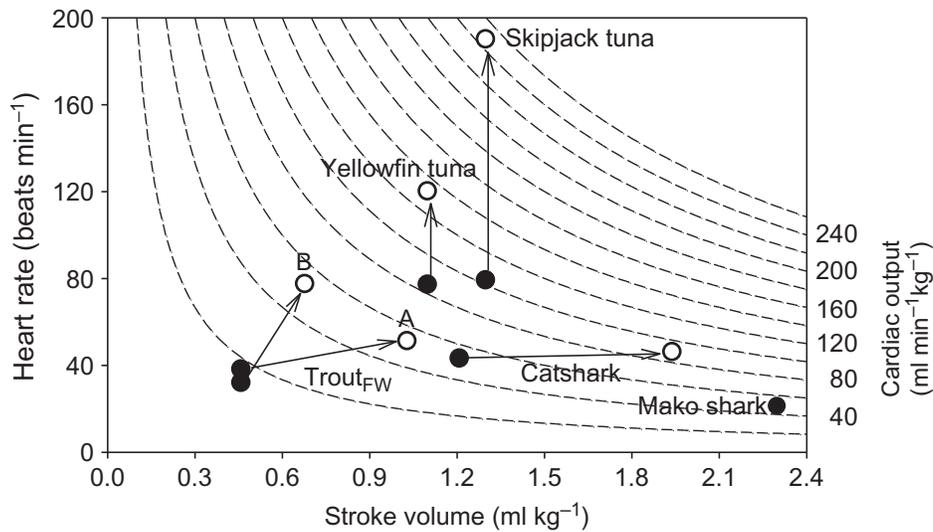


Figure 1.2. Relationship between heart rate, stroke volume, and cardiac output in freshwater adapted rainbow trout (Trout_{FW}), yellowfin and skipjack tunas, catshark, and mako shark. Filled circles show values recorded at resting or routine metabolic rates, and open circles estimated maximal values. In teleost species other than tunas, early studies (Kiceniuk and Jones, 1977) indicated that increases in cardiac output accompanying increases in metabolic rate were accomplished primarily by increases in stroke volume (indicated by data point labeled A). More recent work with using trout not directly wired to recording equipment (Altimiras and Larsen, 2000) showed increases in metabolic rate with activity were accompanied more by increases in heart rate than stroke volume (indicated by data point labeled B). Altimiras and Larsen (2000) did not measure cardiac output, so stroke volume at rest and maximum cardiac output were assumed to equal those measured by Kiceniuk and Jones (1977). We conclude that elasmobranch species (represented by catshark) generally increase cardiac output primarily by increases in stroke volume, although data are limited. In contrast, the predicted cardiac output required to meet skipjack and yellowfin tunas' estimated maximum metabolic rates can be met with observed increases in heart rate, with no concomitant increase in stroke volume. Data for tunas, catshark, and mako shark are from, Bushnell and Brill (1992), Piiper et al. (1977), and Lai et al. (1997), respectively.

Jones, 1977; Kolok and Farrell, 1994). Additionally, more generally in teleosts (other than tunas), the consensus has been that ~40–60% of the increases in cardiac output accompanying increases in $[O_2]_{\text{delivery}}$ are accomplished by increases in SV, with the remainder being due to increases in HR (Farrell, 1991; Farrell and Jones, 1992). Tunas were considered the exception; in that increase in cardiac output (and therefore $[O_2]_{\text{delivery}}$) are accomplished almost solely through increases in heart rate while stroke volumes remain unchanged (Fig. 1.2; Brill and Bushnell, 2001). More recent, work employing data loggers have indicated that cardiac output in teleosts may be modulated more by increases in heart rate than by increases in stroke volume as previously thought (e.g., Armstrong, 1986, 1998; Altimiras and Larsen, 2000; Clark et al., 2005; Iversen et al., 2010). At least in catsharks, increases in cardiac output accompanying increases in metabolic rate (i.e., $[O_2]_{\text{delivery}}$) are accomplished

predominately by increases in stroke volume, with lesser increases in heart rate (Fig. 1.2; Piiper et al., 1977). Furthermore, more generally in elasmobranchs, the consensus is that increases in HR make a minor contribution to increases in cardiac output, with increases $[O_2]_{\text{delivery}}$ accomplished predominately by increases in SV and increases in arterio-venous blood oxygen content difference (Emery, 1985; Scharold et al., 1989; Farrell, 1991; Scharold and Gruber, 1991; Tota and Gattuso, 1996; Carlson et al., 2004). So in at least in this respect (i.e., the relative increases in heart rate and stroke volume accompanying increases in cardiac output) elasmobranchs and teleosts may differ. We note, however, that investigations of the relative increases in heart rate and stroke volume responsible for increases in cardiac output in elasmobranchs are relatively limited, in comparison to the data available for teleosts and encourage investigations in this area.

As with cardiovascular function at routine activity levels, differences in cardiovascular function between teleosts and elasmobranchs become most evident when comparisons are made between the shortfin mako shark and tunas at maximum rates of $[O_2]_{\text{delivery}}$ (~ 500 and ~ 2500 mg O_2 kg^{-1} h^{-1} , respectively; Gooding et al., 1981; Graham et al., 1990; Sepulveda et al., 2007). We contend that this very substantial difference in rates of maximum $[O_2]_{\text{delivery}}$ is primarily related to differences in maximum cardiac output, which, in turn, is a function of maximum achievable HR. Maximum HR is ~ 60 – 70 beats min^{-1} in all shark species studied to date, including the mako shark (Table 1.2; Piiper et al., 1977; Sharold et al., 1989; Graham et al., 1990; Scharold and Gruber, 1991; Lai et al., 1997; Dowd et al., 2006), but above 200 beats min^{-1} in tunas (Brill, 1987; Keen et al., 1995). We also note, however, that a second major difference affecting the differences in maximum $[O_2]_{\text{delivery}}$ between the shortfin mako shark and tunas appears to be gill anatomy. As described by Wegner et al. (2012), the former have greater interlamellar spacing, fewer secondary lamella and a smaller total gas exchange area per unit body mass than the latter, and it is these factors that also likely limit maximum rates of oxygen transfer from the ventilatory water stream to the blood. Because this topic is reviewed by Wegner (2015), it will not be discussed further here.

Increasing $[O_2]_{\text{delivery}}$ could obviously also be accomplished by either an increase in $[O_2]_{\text{arterial}}$ or a decrease in $[O_2]_{\text{venous}}$ (Eq. 1.1). Taking these issues in turn, in at least in three species of elasmobranchs (catshark, spiny dogfish, and lemon shark), measured $[O_2]_{\text{venous}}$ at routine metabolic rates are at levels ($<20\%$ saturation) that likely preclude reduction in $[O_2]_{\text{venous}}$ as major mechanism for increasing oxygen delivery (Lenfant and Johansen, 1966; Baumgarten-Schumann and Piiper, 1968; Hanson and Johansen, 1970; Bushnell et al., 1982). Moreover, using Eq. 1.1 and data on the metabolic rate, cardiac output, $[O_2]_{\text{arterial}}$, and blood oxygen dissociation curves for epaulette shark and eastern shovelnose ray presented in Speers-Roesch et al. (2012a),

calculated $[O_2]_{\text{venous}}$ is $\sim 10\%$ saturation with an oxygen partial pressure (PO_2) of < 2 kPa. We do note that catsharks and the leopard shark (*Triakis semifasciata*) have been reported to maintain a significant $[O_2]_{\text{venous}}$ at rest (Butler and Taylor, 1975; Short et al., 1979; Lai et al., 1990a). Although in the latter species, $[O_2]_{\text{venous}}$ falls to $< 20\%$ saturation at modest levels of activity; and in the mako shark the available data on the existence of functional $[O_2]_{\text{venous}}$ are at least equivocal (Lai et al., 1990a, 1997).

Likewise, the ability for elasmobranchs to increase $[O_2]_{\text{delivery}}$ through an increase $[O_2]_{\text{arterial}}$ appears not to be universal, although once again available data are limited. Bushnell et al. (1982) measured an increase in $[O_2]_{\text{arterial}}$ with exercise in lemon shark, which occurred through a combination of an increase in hematocrit (assuming there is no significant red blood cell swelling) and arterial PO_2 . An approximate doubling (or more) from a normal hematocrit of $\sim 20\%$ (Table 1.1) to 34–50% has been recorded in mako shark following strenuous exercise associated with hook-and-line capture (when they would presumably be recovering from exhaustive activity and would have elevated metabolic rates) (Emery, 1986; Wells et al., 1986; Hight et al., 2007). However, neither the blacktip reef shark (*Carcharhinus melanopterus*) nor the giant shovelnose ray (*Rhinobatos typus*) shows an increase in hematocrit in response to exercise (Chopin et al., 1998). Other investigators have likewise concluded that elasmobranchs lack the mechanisms for significantly increasing hematocrit through splenic contracture that are present in teleosts (Opdyke and Opdyke, 1971; Nilsson et al., 1975; Yamamoto et al., 1981; Yamamoto and Itazawa, 1989; Lowe et al., 1995). Consistent with this latter observation are the hematocrit values reported for a number of shark species following capture by fishing gear. In these instances hematocrits are close to, or not significantly different from, normal values ($\sim 20\text{--}30\%$) and are always below 40% (Mandelman and Farrington, 2007; Brill et al., 2008; Frick et al., 2010; Marshall et al., 2012).

The elevated hematocrit of shortfin mako and great white sharks (reported range: 22–60% and 22–49%, respectively) compared to other shark species (reported range: 9–33%) (Larsson et al., 1976; Emery, 1985, 1986; Filho et al., 1992) does, in turn, raise questions concerning blood viscosity. The positive impact of increasing hematocrit (i.e., $[O_2]_{\text{arterial}}$) on $[O_2]_{\text{delivery}}$ could potentially be negated by concomitant increases in blood viscosity. This can increase blood pressure and the work load of the heart, and thus decrease maximum cardiac output (Wells and Baldwin, 1990). Blood viscosity, moreover, increases nonlinearly with hematocrit, whereas $[O_2]_{\text{arterial}}$ increases linearly. Blood oxygen transport capacity (i.e., the ratio of $[O_2]_{\text{arterial}}$ to blood viscosity) is therefore a nonlinear function of hematocrit with the point of maximum blood oxygen transport capacity indicating the optimal hematocrit (Fletcher and Haedrich, 1987). Based on

changes in $[O_2]_{\text{arterial}}$ and viscosity occurring with changes in hematocrit, it appears that neither teleosts (Wells and Baldwin, 1990; Wells and Weber, 1991) nor elasmobranchs (Baldwin and Wells, 1990) function at their optimal hematocrit. We also note, however, that under equivalent measurement conditions (i.e., at equal hematocrit and the same temperature), elasmobranch blood appears to have dynamic (or shear rate) viscosity at the upper end of the range of those observed in teleosts, possibly due to the high levels of urea and trimethylamine oxide in elasmobranch plasma forming protein–solute interactions (Baldwin and Wells, 1990; Brill and Jones, 1994). It is therefore at least theoretically possible that blood acidosis following exhaustive exercise (such as that associated with capture by fishing gear) in elasmobranchs could increase blood viscosity (by effecting such protein–solute interactions) sufficiently to compromise cardiac output and $[O_2]_{\text{delivery}}$ and thus to prolong, or even to preclude, recovery. To the best of our knowledge, the effects of plasma pH on elasmobranch blood viscosity have never been measured, although this could be a fruitful area of investigation. We also note that elasmobranchs exhibit increases in circulating catecholamines following exposure to stressful conditions (e.g., hypoxia, burst swimming, etc.), which increase cardiac heart rate and force of contraction (Van Vliet et al., 1988; described more fully in Section 3.1.1). These cardiac responses, in turn, could counteract the effects of increases in blood viscosity.

2.2. Responses to Hypoxia

Coastal zones and tidal estuaries serve as important habitats and critical nursery areas for elasmobranchs (e.g., Castro, 1993; Conrath and Musick, 2010; Espinoza et al., 2011). Primarily because of anthropogenic activity, however, the frequency of occurrence, severity, and spatial scale of episodic coastal hypoxia are increasing worldwide (Diaz and Rosenberg, 2008; Diaz and Breitburg, 2009). The volume of the hypoxic water in the world’s oceans is also predicted to increase dramatically due to increasing ocean temperatures associated with global climate change (Stramma et al., 2010; Deutsch et al., 2011). For these reasons alone, we consider that research to gain a better understanding of the effects of hypoxia on a range of elasmobranch species is clearly warranted.

We also posit, however, that the species-specific tolerances of hypoxia are informative with respect to the diversity of elasmobranch cardiovascular physiology. We note that at least six elasmobranch species enter severely hypoxic areas as part of their foraging strategies: cownose rays, *Rhinoptera bonasus*, in the Gulf of Mexico (Craig et al., 2010); scalloped hammerhead, *Sphyrna lewini*, in the Gulf of California (Jorgensen et al., 2009; Bessudo et al., 2011); shortfin mako shark in the eastern Pacific Ocean (Vetter et al., 2008); Atlantic stingray, *Dasyatis sabina*, in seagrass meadows

(Dabruzzi and Bennett, 2013); bigeye thresher shark, *Alopias superciliosus*, and six gill shark, *Hexanchus griseus*, in the Pacific Ocean (Nakano et al., 2003; Weng and Block, 2004; Musyl et al., 2011; Coffey and Holland, pers. comm.); and whale shark, *Rhincodon typus*, in the eastern central Atlantic Ocean (Escalle et al., 2014). In addition, at least two species, the torpedo ray (*Torpedo marmorata*) and epaulette shark (*Hemiscyllium ocellatum*), are routinely subjected to extended periods of severely hypoxic conditions. The former when they become trapped in tide pools (Hughes and Johnston, 1978) and the latter at night in its Australian coral reef flat environment when isolation from the surrounding water mass, and respiration of coral, algae, and other reef inhabitants, reduce ambient oxygen levels to below 10% air saturation (Kinsey and Kinsey, 1967; Renshaw et al., 2002). The epaulette shark is the best studied of this group primarily because its relatively small size and ease of acquisition make it highly tractable for use in controlled laboratory studies (e.g., Dowd et al., 2010; Speers-Roesch et al., 2012a,b; Hickey et al., 2012).

Although available data are from studies on a relatively limited number of species (compared to those on teleosts), elasmobranchs acutely exposed to hypoxia generally exhibit:

1. bradycardia accompanied by small (if any) decreases in cardiac output because of the concomitant increase in stroke volume (e.g., Piiper et al., 1970; Butler and Taylor, 1975; Speers-Roesch et al., 2012b), or heart rate and stroke volume decreasing simultaneously (e.g., Sandblom et al., 2009);
2. increases in frequency and amplitude of ventilatory movements; with a concomitant increase in ventilation volume accompanied by a decrease in utilization (e.g., Hughes, 1978; Carlson and Parsons, 2003); and
3. an increase in swimming speed and mouth gape in ram ventilating sharks, or a decrease in activity in sedentary species (e.g., Parsons and Carlson, 1998; Carlson and Parsons, 2001).

At this gross level, the responses of elasmobranchs to hypoxia appear largely similar to those of teleosts. Closer examination, however, reveals important differences. As we will describe subsequently (see Section 3.1.1), elasmobranchs have limited direct adrenergic vascular innervation. Circulating catecholamines released from chromaffin tissue can, however, affect vasoconstriction (through α -adrenergic receptors) and therefore increase venous return. This, in turn, augments stroke volume thus maintaining cardiac output during hypoxic bradycardia. In spiny dogfish (*S. acanthias*), hypoxia can be (but not always is) accompanied by increases in circulating catecholamines (Butler et al., 1978; Perry and Gilmour, 1996) and changes in venous capacitance (Sandblom et al., 2009). This may explain the fall in cardiac output accompanying hypoxic bradycardia observed in this species. Whether this situation is unique to spiny dogfish, or whether (as proposed by Sandblom et al., 2009) it represents

fundamental differences between teleosts and elasmobranchs is currently unknown. Also elasmobranchs generally have lower resting hematocrits than do teleosts (Fänge, 1992) and, more importantly, they do not evince an increase hematocrit in response to hypoxia as do teleosts (Butler et al., 1979; Perry and Gilmour, 1996; Short et al., 1979; Carlson and Parsons, 2003); and this includes the hypoxia-tolerant epaulette shark (Routley et al., 2002). Gray carpet shark (*Chiloscyllium punctatum*) do show an increase hematocrit in response to anoxia, but it is unknown if this is due to splenic contracture (as it is in teleosts; e.g., Yamamoto and Itazawa, 1989; Pearson and Stevens, 1991) or due to hemoconcentration resulting from net fluid transfer out of the circulatory system (Chapman and Renshaw, 2009). The ability of the elasmobranch spleen to function as a red blood cell reservoir, and to contract and release red blood cells in response to an increase in circulating catecholamines, remain controversial (Opdyke and Opdyke, 1971; Nilsson et al., 1975).

Elasmobranchs, moreover, exhibit a range of tolerances of hypoxia, with some species showing extreme hypoxia tolerance evinced by oxygen levels where impairment to neurological function becomes apparent (e.g., loss of equilibrium, righting reflex, rhythmic swimming, and ventilatory movements) and muscle spasms commence. For example, the level of extreme hypoxia inducing neurological impairments is ~ 2 kPa or $\sim 10\%$ saturation in Atlantic stingray (*Dasyatis sabina*) and torpedo ray (Chapman et al., 2011; Dabruzzi and Bennett, 2013; Hughes, 1978; Hughes and Johnston, 1978; Speers-Roesch et al., 2012a) and at ~ 1 kPa or 5% air saturation in epaulette shark (Wise et al., 1998). In the case of epaulette shark, the ability to withstand hypoxia evolved in response to the strong selective pressures exerted by the severely hypoxic conditions occurring in their particular coral reef environments during nocturnal spring low tides (Renshaw et al., 2002; Dowd et al., 2010). More specifically, epaulette shark show both neuronal protective responses and cardio-respiratory adaptations; we will only briefly describe the former, however, as they are not germane to the thrust of this chapter. During severe hypoxia epaulette shark evince hypometabolism at least in brain areas associated with neurons that exert motor function and cardiorespiratory control, but not in brain areas associated with input from electroreceptors and lateral line (Mulvey and Renshaw, 2000). The brain areas showing hypometabolism demonstrate an increase in levels of adenosine, which initiates metabolic depression (Renshaw et al., 2002), and heterogenous redistribution of γ -aminobutyric acid (GABA), which presumably has a neuroprotective function (Mulvey and Renshaw, 2009). The epaulette shark also does not experience neuronal apoptosis in response to prolonged hypoxia (Renshaw and Dyson, 1999). In spite of marked hypotension, and a reduction in cardiac output (described below), epaulette sharks do not show a reduction cerebral blood flow during two hours of severe hypoxia (~ 1 kPa or 5% air

saturation at 24°C) due to cerebral vessel dilation resulting from an increased production of nitric oxide (mechanism described in [Section 4.2](#); [Renshaw and Dyson, 1999](#); [Söderström et al., 1999](#)). Moreover, although cerebral blood vessels do respond to applied adenosine, as they do in other vertebrates, adenosine does not appear to be involved in the maintenance of cerebral blood flow during hypoxia ([Söderström et al., 1999](#)).

The cardiovascular system of epaulette shark likewise shows specific adaptations for surviving sustained hypoxia. Based on oxygen level (P_{crit}) at which epaulette shark transition from being oxyregulators (i.e., where the aerobic metabolic rate remains independent of ambient oxygen) to being oxyconformers (i.e., where the aerobic metabolic rate falls in concert with reductions in ambient oxygen), this species is more hypoxia tolerant than the eastern shovel nose ray, which also occupies Australian reef environments, but which is not regularly exposed to hypoxia ([Speers-Roesch et al., 2012b](#)). Both species maintain cardiovascular function (heart rate, stroke volume, cardiac power output, and dorsal aortic blood pressure) until ambient oxygen levels reach or fall slightly below their respective P_{crit} values. But since the P_{crit} of the epaulette shark is below that of the shovel nose ray, cardiovascular function is maintained to lower oxygen levels evincing the former species' better tolerance of hypoxia ([Speers-Roesch et al., 2012b](#)). Moreover, repeated exposure to hypoxia reduces aerobic metabolic rate in epaulette sharks which, in turn, increases tolerance to hypoxia (i.e., reduces P_{crit}) ([Routley et al., 2002](#)). It is unknown, however, if this response is extant in other elasmobranch species. In addition, cardiac tissue of the epaulette shark likewise shows adaptations to survive severe and extended hypoxia (as does the brain neural tissue). Specifically, the mitochondria isolated from ventricular tissue of epaulette demonstrate a significantly better ability to maintain stability and integrity during hypoxia than do those isolated from ventricular tissue of the less hypoxia tolerant eastern shovel nose ray. This ability is primarily due to a depressed free radical release ([Hickey et al., 2012](#)). But it is unknown if this particular adaptation occurs in other hypoxia tolerant elasmobranch species.

The third factor determining P_{crit} (i.e., the ability to maintain blood oxygen delivery to tissues during hypoxia) is blood oxygen binding affinity; more specifically the P_{50} (i.e., the PO_2 when blood is half saturated). A higher blood oxygen binding affinity (i.e., a low P_{50}) helps maintain higher $[O_2]_{\text{arterial}}$ during hypoxia than in fish with lower blood oxygen affinity (i.e., blood with a higher P_{50}). A two-species comparison of blood oxygen affinity between epaulette shark and the less hypoxia tolerant eastern shovel nose ray implies a correlation between P_{crit} (a measure of hypoxia tolerance) and P_{50} (a measure of blood oxygen affinity) ([Speers-Roesch et al., 2012a](#)). A more extensive examination of the P_{crit} and blood P_{50} values of broader range of elasmobranch species ([Table 1.3](#)) shows a broad range of blood

Table 1.3

Critical oxygen levels (i.e., the lowest oxygen level where aerobic metabolic rate can be maintained in resting fish) and blood P₅₀ (the partial pressure of oxygen required to achieve 50% blood oxygen saturation) in elasmobranchs

| Species | Blood P ₅₀ | Critical oxygen level | Temperature | References |
|--|-----------------------|---|--------------------|--|
| Epaulette shark, (<i>Hemiscyllium ocellatum</i>) | 4.3 kPa ^a | 5.1 kPa, 25% saturation | 28°C | Speers-Roesch et al. (2012a) |
| Epaulette shark | | 6.5 kPa, 32% saturation ^b | 25°C | Routley et al. (2002) |
| Cat shark (<i>Scyliorhinus canicula</i>) | 2.9 kPa ^b | 8.0 kPa, 38% saturation 10.7 kPa, 70% saturation | 7 and 12°C 17°C | Butler and Taylor (1971) Pleschka et al. (1970) Butler and Taylor (1975) |
| Cat shark (<i>Scyliorhinus stellaris</i>) | 2.3 kPa ^c | between 7–13 kPa, 35–61% saturation | 15–17°C | Piiper et al. (1970) Piiper and Baumgarten-Schumann (1968) |
| Bamboo shark (<i>Hemiscyllium plagiosum</i>) | | 6.6 kPa, 30% saturation | 23°C | Chan and Wong (1977) |
| Bonnethead shark (<i>Sphyrna tiburo</i>) | 1.9 kPa | 9.4 kPa, 46% saturation | 28°C | Haggard (2000) (cited in Carlson and Parsons, 2003) |
| Shovelnose ray (<i>Aptychotrema rostrata</i>) | 6.3 kPa ^d | 7.2 kPa, 35% saturation | 28°C | Speers-Roesch et al. (2012a) |
| Sandbar shark <i>Carcharhinus plumbeus</i> | ~2.8 kPa ^e | | 25°C | Brill et al. (2008) |
| Torpedo ray <i>Torpedo marmorata</i> | 2.7 kPa ^f | ~6.6 kPa, ~30% saturation | 15°C | Hughes (1978) |
| Thornback ray <i>Raia oscillata</i> | 4.0 kPa ^g | | 15°C | Hughes and Wood (1974) |

^a28°C, P_{CO2} ~0.23 kPa, pH ~7.8

^b17°C, P_{CO2} 0.3 kPa, pH 7.58

^c17°C, P_{CO2} 0.2 kPa

^d28°C, P_{CO2} ~0.23 kPa, pH ~7.8

^e25°C, P_{CO2} 0.37 kPa, pH ~7.8

^fpH 7.8

^gpH 7.7

^hNot preconditioned by previous exposure to hypoxia.

oxygen affinities (P_{50}) and hypoxia tolerances (P_{crit}), but not necessarily a strict correlation between the two. We do note, however, that blood oxygen affinity data were collected over a broad range of temperature, pH and carbon dioxide levels (Table 1.3), which makes direct inter-species comparison somewhat tenuous. The high blood oxygen affinities (i.e., low P_{50}) of several of the elasmobranch species implies, however, that tolerance of hypoxia may be wide spread in elasmobranchs, albeit with species-specific differences.

Based on these observations, and the number of elasmobranch species that routinely subject themselves to hypoxic areas as part their normal foraging behavior described above, we argue that this demonstrates the evolutionary plasticity of the elasmobranch cardiorespiratory system and implies there is likely to be a broad range of tolerance and physiological responses to hypoxia in elasmobranchs, but that remain largely undescribed because of the relative paucity of species studied to date. We speculate that some, or all, of the adaptations shown by epaulette shark probably occur in other hypoxia-tolerant elasmobranch species, but these remain to be described. We contend that (i) these questions are of now of more than of academic interest because (as noted above) of increasing frequency of occurrence and severity of coastal hypoxic zones and effects of directional global climate; and (ii) the cardiovascular consequences of hypoxia need to be investigated in a broader range of elasmobranch species, both in terms of phylogeny and ecomorphotypes, in order to predict their ability to function under increasingly hypoxic conditions that many are now facing. We note majority of studies using epaulette shark used only one comparative species – the shovel nose ray – and encourage investigations in this area employing a broader range of elasmobranch species. Although we readily admit that this may be difficult because of generally limited access to live elasmobranch species in captivity.

2.3. Elasmobranch Cardiac Anatomy

The elasmobranch heart (as in other fishes) is composed of four chambers contained within the pericardium: sinus venosus, atrium, ventricle, and an outflow tract – the conus arteriosus (in some respects functionally equivalent to the bulbus arteriosus in teleosts) (Butler and Metcalfe, 1988). The anatomy, functional aspects, and vascularization of the various chambers are extensively delineated elsewhere (e.g., Tota, 1983, 1989, 1999; De Andrés et al., 1990a,b; Tota and Gattuso, 1996; Farrell et al., 2012), therefore an extensive description will not be repeated here. Rather, as in the previous section, we will attempt to gain insight into the functional characteristics of the elasmobranch heart (and the larger implications with respect to whole animal physiological capabilities and tolerances) by broadly comparing ventricular anatomy, physiology, and functional characteristics across elasmobranch species.

As in other vertebrates, elasmobranch myocardial cells are organized into either:

1. a crisscrossed mesh forming thin myocardial bundles (trabeculae), which in turn are interlaced into a complex network resulting in the myocardium having a spongy appearance at both the macroscopic and microscopic levels (Tota, 1989; Tota and Gattuso, 1996); or
2. an aligned and orderly arrangement of dense and parallel myocardial bundles that encircle the ventricle in various directions (described in detail by Sanchez-Quintana and Hurle, 1987).

The former is generally referred to as the “spongiosa” or “spongy myocardium,” and the latter as the “compacta,” or “compact myocardium” (Tota and Gattuso, 1996). We will employ the latter terms for both as they are more descriptive.

In fishes, the ventricular wall is composed of various relative amounts of spongy and compact myocardium ranging from the ventricle being made up of only the former, to the latter comprising >30% of the ventricular wall cross-sectional area. Based on this characteristic, and the vascularization of the spongy and compact myocardium, fish ventricles are classified into four types (usually designated by the Roman numerals I–IV); although the types represent a continuum rather than discreet categories. The characteristics of each subtype are described in detail by Tota (1989), Davie and Farrell (1991b), Tota and Gattuso (1996), and Farrell et al. (2012). In brief,

Type I – ventricle comprised of only avascular spongy myocardium;

Type II – ventricle comprised of an inner avascular spongy myocardium overlain with a thin layer of compact myocardium containing coronary blood vessels (i.e., with an arterial blood supply);

Type III – ventricle comprised of substantial (but <30% of the ventricular cross-sectional area) compact myocardium containing coronary blood vessels, with capillary vascularization of the spongy myocardium and thebesian vessel-like shunts connecting the arterial oxygenated coronary blood vessels to the venous lacunary system of the spongy myocardium (at least in elasmobranchs);

Type IV – ventricle with a thicker (>30% of the ventricular cross-sectional area) compact myocardium containing coronary blood vessels, and a vascularized spongy myocardium also with thebesian vessel-like shunts.

In teleosts, Type I ventricle morphology appears to be the most common (estimated to be present in 50–80% of extant species, Farrell et al., 2012) and

is generally exemplified by hearts found in sluggish benthic species (e.g., flat fishes such as *Pleuronectes* spp.). Type II hearts are present in more active species, such as rainbow trout. Type III and IV ventricle morphologies are present in the most active teleost species (e.g., the tunas) (Santer and Greer Walker, 1980; Santer, 1985; Farrell et al., 2012). In contrast, all elasmobranch species studied to date have ventricle morphologies classified as Type III or IV (Tota, 1989; Tota and Gattuso, 1996; Farrell et al., 2012). The selective pressures favoring development of exclusively Type III and Type IV ventricles in elasmobranchs are unknown, but have been previously ascribed to factors as diverse as the energetic cost of viviparity, and sharks being negatively buoyant and therefore their need to maintain hydrodynamic lift by continuous forward motion. These, in turn, result in the need for a more efficient cardiac pump (Santer and Greer Walker, 1980; Tota, 1983, 1989). The above hypotheses do not, however, explain the presence of Type III and IV ventricle morphologies in males, or their presence in the benthic batoid elasmobranch species.

We therefore offer an alternative argument which we contend may better explain the apparent universality of Type III and IV ventricle morphologies in elasmobranchs: the necessity of an arterial blood supply to the spongy myocardium due to the potential for very low venous blood PO_2 . In Type I and II ventricle morphologies, the oxygen supply to the spongy myocardium is exclusively from the luminal venous blood. Whereas in Type III and IV ventricle morphologies, both the spongy and compact myocardium receive an arterial blood supply with $[O_2]_{\text{arterial}}$ and PO_2 levels much higher than those in the venous blood contained within the ventricular chamber (De Andrés et al., 1990a,b). Because the ventricle lumen receives the full cardiac output (i.e., venous return), and the ventricle mass is generally $<0.1\%$ of the body mass, the blood flow per unit mass of ventricular tissue is huge. Therefore, even if $[O_2]_{\text{venous}}$ is low, the rate of oxygen delivery (i.e., $[O_2]_{\text{venous}} \times$ rate of blood flow per unit ventricle mass) provided by the luminal venous blood would be very high and therefore should be able to support a significant rate of ventricular aerobic energy production (Cox et al., manuscript in preparation). We note, however, that it is blood PO_2 that provides the driving gradient for moving O_2 into the myocardium. Jones (1986) concluded that a luminal (i.e., venous blood) PO_2 of at least 1.3 kPa is the minimum needed for adequate rates of O_2 diffusion into myocardial cells. But a number of elasmobranchs (e.g., catsharks, lemon, leopard, and epaulette sharks, and eastern shovelnose ray) have venous PO_2 that is at or below this value at routine activity levels (Piiper et al., 1977; Bushnell et al., 1982; Taylor and Barrett, 1985; Lai et al., 1990a). This implies that an arterial blood supply to the spongy myocardium (which is one of the defining characteristics of Type III and Type IV hearts) is

necessary for the maintenance of cardiac power output. Other evidence supportive of our contention are the observations that:

1. maximum cardiac power output is reduced by 64% when isolated spiny dogfish hearts specifically deprived of coronary circulation are perfused with a severely hypoxic ($PO_2 = 1$ kPa) Ringer's solution (Davie and Farrell, 1991a);
2. cardiac power output decreases with increasing levels of hypoxia in epaulette shark and eastern shovelnose ray in concert decreases in arterial blood O_2 saturation (Speers-Roesch et al., 2012a,b), which are presumably accompanied by reductions in venous PO_2 (although the latter was not measured).

We note, however, that is also possible that the selective pressure for development of Type III and Type IV ventricles in elasmobranchs are more related to the low venous PO_2 s that occur during times of elevated metabolic O_2 demand (described above).

One of the characteristics used to classify hearts as Type III or IV is the fraction of the ventricle cross-sectional areas made up of compact myocardium. In addition, it has been assumed (e.g., Farrell et al., 2012) that Type IV ventricles are associated with higher rates of oxygen and metabolic substrate delivery rates to the tissues in species such as the tunas and mako shark, which reflect the convergent evolution in regionally endothermic teleosts and elasmobranchs (Bernal et al., 2001). As discussed in the previous section, the evidence does not support the conclusion that thermoconserving lamnid species (e.g., shortfin mako shark) have larger hearts than the nonthermoconserving pelagic species, with the possible exception of the great white shark (Fig. 1.1). Likewise, the evidence is equivocal that the fraction of the ventricle composed of compact myocardium is greater in thermoconserving teleosts (Fig. 1.3A) and elasmobranchs (Fig. 1.3B). When comparing data just across the Selachii (i.e., shark species), the fraction of the ventricle composed of compact myocardium is higher in at least the three thermoconserving species (gray shaded bars, Fig. 1.3B) and is above the 30% compact myocardium characteristic that distinguishes Type III from Type IV hearts. The implication being that one or more of the adaptations required for regional endothermy (e.g., the presence of vascular counter-current heat exchangers) (Brill et al., 1994; Patterson et al., 2011) increase total peripheral resistance, and therefore ventral aortic pressure and the power demand of the ventricle, which is the product of cardiac output and mean ventral aortic pressure. In addition, mean ventral aortic pressure in mako shark (6.7 kPa, Table 1.1) is above that of spotted catshark (5.3 kPa, Table 1.1), spiny dogfish, smooth dogfish (*Mustelus canis*), and Port Jackson shark (2.8–4.2 kPa) (Satchell, 1999). We also note,

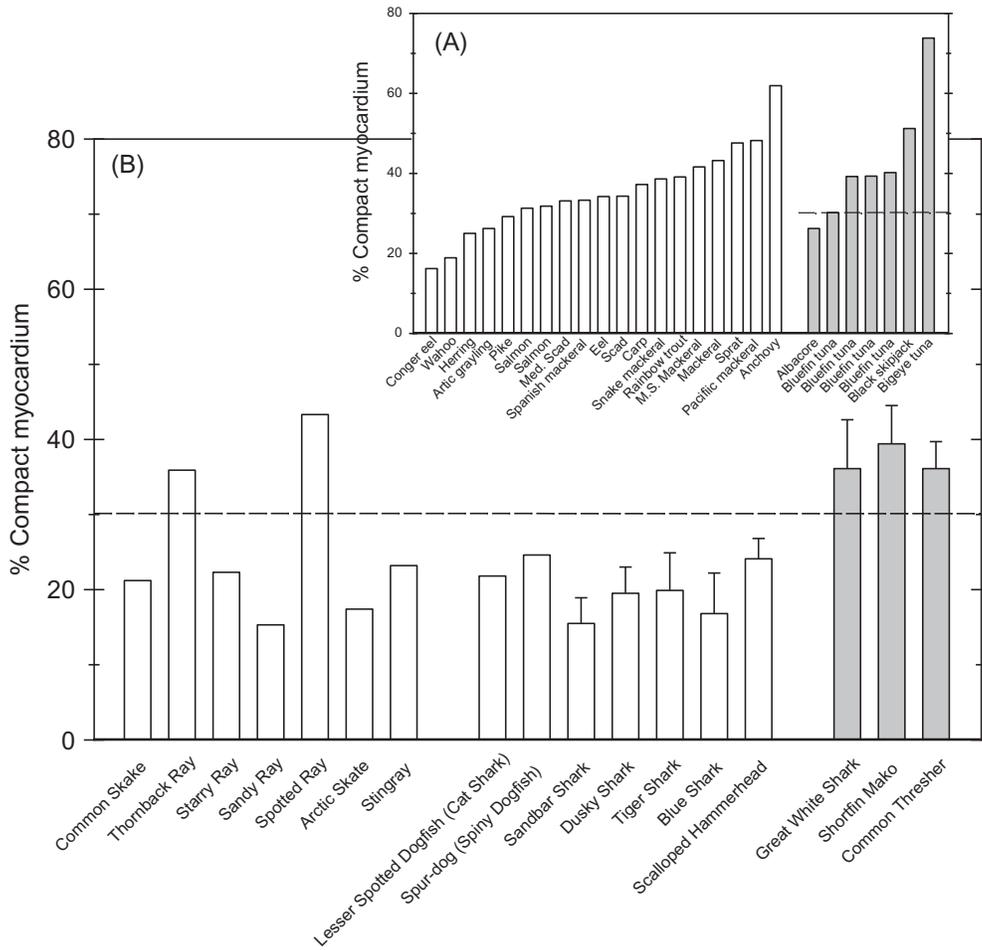


Figure 1.3. The fraction of the ventricle composed of compact myocardium in teleost (A) and elasmobranch (B) species. Shaded bars indicate thermoconserving (i.e., regionally endothermic) species. The horizontal dashed line shows the 30% value for the fraction of the ventricle composed of compact myocardium that is considered to differentiate Type III from Type IV ventricles (Tota 1983, 1989; Davie and Farrell, 1991b). Teleost data (excluding tunas) are from Table 1.2 (p. 24) in Santer (1985) and common names match those listed in that table. Tuna data are from Tota (1978), Tota et al. (1983) and Sanchez-Quintana and Hurlle (1987). Elasmobranch data are from Emery et al. (1985). Error bars on the elasmobranch data are \pm SEM.

however, the presence of a thick compact myocardium (36–43% of the cross sectional areas, Type IV hearts) in at least two species of Batoids, the thornback (*Raja clavata*) and spotted rays (*Raja montagui*) (Fig. 1.3B), neither of which are known to be regional endotherms. We therefore argue that the selective pressures resulting in the development of a thick compact myocardium in both teleosts and elasmobranchs remain unclear. Also, the capillary geometry of the spongy and compact myocardial layers remains undescribed in elasmobranch hearts, and a comparative study in this area would be insightful.

2.4. Cardiac Metabolic Biochemistry

Cardiac metabolic biochemistry in teleosts and elasmobranchs has been described in detail by [Driedzic \(1992\)](#), [Dickson et al. \(1993\)](#), [Dickson \(1995\)](#), [Ballantyne \(1997\)](#), [Bernal et al. \(2003b\)](#), [Gamperl and Driedzic \(2009\)](#), and [Speers-Roesch and Treberg \(2010\)](#), so only a cursory discussion will be provided here. Sustained oxygen and metabolic substrate delivery to the tissue by the cardiovascular system requires an ongoing match of cardiac power demand (which is a function of mean ventral aortic pressure and cardiac output) and rates of metabolic ATP production. The latter is, in turn, a function of oxygen and metabolite supply to the cardiac muscle itself (discussed above), myocardial efficiency, and myocardial enzymatic activity ([Davie and Franklin, 1992](#)). To briefly explore the latter we will concentrate on three enzymes: citrate synthase (CS), pyruvate kinase (PK), and lactate dehydrogenase (LDH). CS catalyzes the first reaction in the Krebs citric acid cycle. Its activity correlates with tissue mitochondrial density. It is therefore an indicator of aerobic capacity, maximal power production, and the ability of tissues to utilize blood borne glucose. PK catalyzes the conversion of phosphoenolpyruvate to pyruvate with the concomitant production of ATP from ADP, which is the final step in glycolysis. It is therefore an indicator of glycolytic capacity. LDH catalyzes the reversible reaction of pyruvate + NADH \rightarrow lactate + NAD⁺ (i.e., the conversion of pyruvate to lactate during anaerobic metabolism or the conversion of lactate to pyruvate for subsequent oxidation) ([Driedzic, 1992](#); [Dickson et al., 1993](#)). Based on available data, it appears that fatty acid (i.e., lipid) metabolism makes a minor, if any, contribution to power production in elasmobranch hearts; unlike the situation in teleosts, especially tunas ([Driedzic, 1992](#); [Moyes, 1996](#); [Speers-Roesch and Treberg, 2010](#)). Fatty acid metabolism will therefore not be discussed further. Elasmobranch hearts do, however, appear to have the ability to metabolize ketone bodies produced by the liver from fatty acids especially during starvation ([Moyes et al., 1990](#); [Speers-Roesch et al., 2006](#); [Speers-Roesch and Treberg, 2010](#)).

As discussed previously, when comparing representative elasmobranch and teleost species (e.g., catsharks and rainbow trout) there are no functional characteristics of the cardiovascular system distinguishing the two groups, and this includes cardiac power demand ([Table 1.1](#)). The mako shark has higher cardiac output and ventral aortic pressure, but because of the larger ventricle mass ([Emery et al., 1985](#)), cardiac power demand per unit ventricle mass is only about twice that of the catshark ([Table 1.1](#)). A similar situation occurs when comparing rainbow trout and tunas. The relative difference of cardiac power demand per unit ventricle mass ($\sim 3.5:1$, [Table 1.1](#)) between the two species is lower than the ratio of cardiac power demand per unit body mass ($> 10:1$, [Brill](#)

and Bushnell, 2001) because of tunas' relatively larger hearts. The overall conclusion being that, when comparing across species, the increase cardiac power production per unit body mass is a result of a combination of the ~ 2.5 times larger heart mass and increased power production per unit ventricle mass (Brill and Bushnell, 2001; Bernal et al., 2003b).

The increased power production per unit ventricle mass is reflected in myocardial enzyme activities. Comparing representative enzyme activity data for rainbow trout and skipjack and yellowfin tunas (Fig. 1.4), it's apparent that the tunas' higher cardiac energy production is reflected in higher activities of CS, PK, LDH per unit weight. In contrast, the situation is less clear when comparisons of myocardial enzymatic activities are made between elasmobranch species, in part because comprehensive cardiovascular data are not available. Nonetheless, activities of CS, PK, LDH per unit weight do not appear universally higher in thermoconserving elasmobranch species (gray cross hatched bars, Fig. 1.4) compared to other elasmobranchs (gray bars, Fig. 1.4). We argue that the lack of clear inequalities between elasmobranch species, versus the clear disparity between tunas and other teleosts, reflects the smaller difference in cardiac energy production per

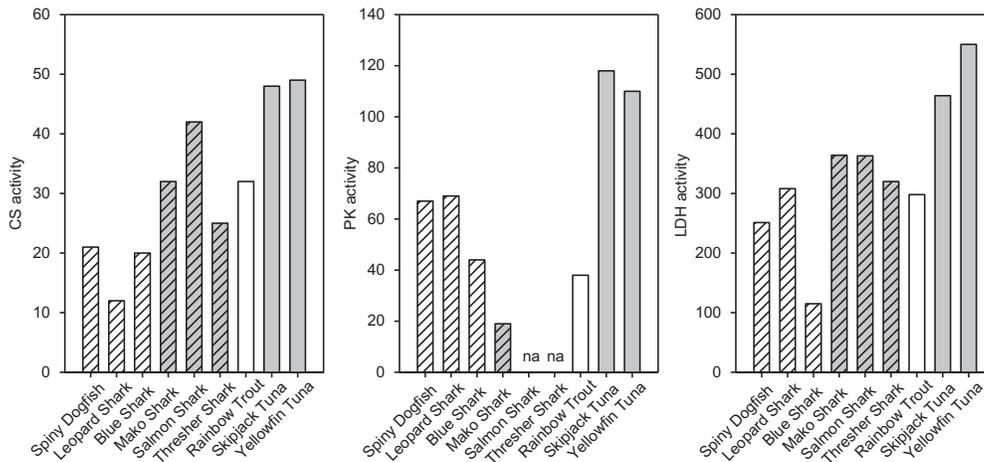


Figure 1.4. Enzyme activities (LDH, lactate dehydrogenase; CS, citrate synthase; PK, pyruvate kinase) in myocardial tissues from elasmobranch and teleost species expressed as μmol substrate converted to product per min per gram of tissue. Cross hatched bars indicate elasmobranch species, gray bars show thermoconserving species (“na” indicates there is no data available). Assay temperatures (spiny dogfish 15°C ; leopard, blue, mako, salmon, and thresher sharks 20°C ; rainbow trout $22\text{--}25^{\circ}\text{C}$; skipjack and yellowfin tunas 25°C) reflect those normally occupied by the various species. We have not corrected enzyme activity data to a common temperature to allow enzyme activities to be compared to rates of cardiac power production (Table 1.1), which were likewise not corrected to a common temperature. Data are from Brill and Bushnell (1991), Driedzic (1992), Dickson et al. (1993), Bernal et al. (2003), and Swimmer et al. (2004).

unit heart mass. This, in turn, reflects smaller differences in cardiac output and mean ventral aortic pressure (Table 1.1) between elasmobranch species (e.g., catshark vs. mako shark) than between teleost species (e.g., rainbow trout vs. tuna).

3. FACTORS CONTROLLING AND EFFECTING CARDIOVASCULAR FUNCTION

Extensive reviews on mechanisms influencing or controlling cardiovascular function in fishes are available in earlier volumes of this series (e.g. Bushnell et al., 1992; Burselson et al., 1992; Olson, 1992; Taylor, 1992) and have been summarized more recently by Olson and Farrell (2006). Fig. 1.5 presents a simplified schematic diagram showing the interactions of factors influencing cardiac output in elasmobranchs, although it is in large measure also applicable to other vertebrates. In the previous section we considered differences in stroke volume and heart rate (and therefore cardiac output and rates of oxygen and metabolic substrate delivery) across elasmobranch species. In this section we will deal with the lower parts of Fig. 1.5, exclusive of the effects of temperature and ambient oxygen.

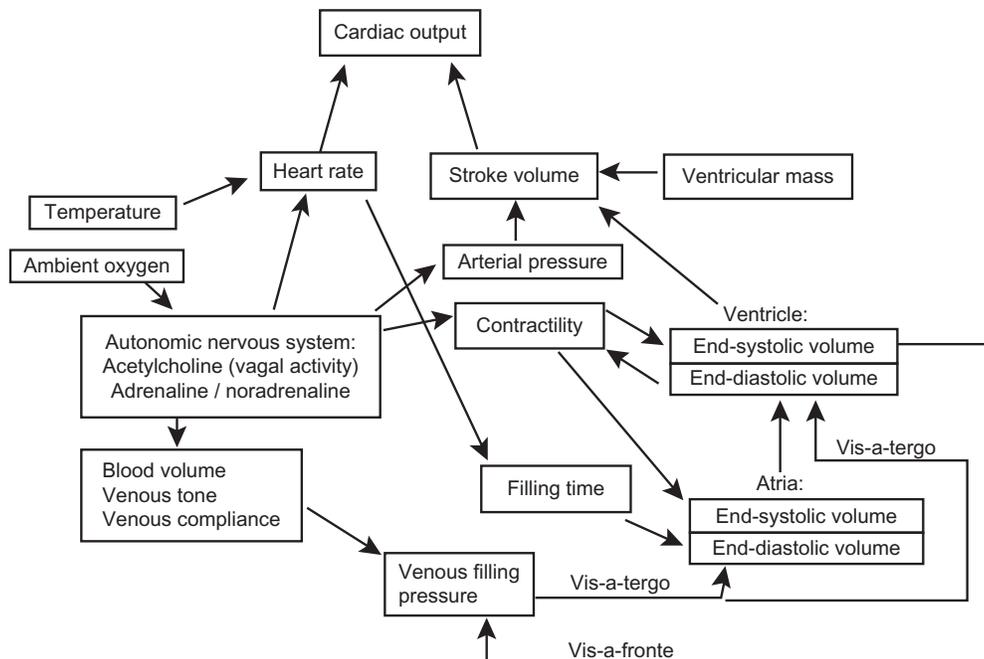


Figure 1.5. Schematic diagram of factors effecting cardiac output in elasmobranchs (redrawn from a figure presented by Farrell, 2001). Negative feedback mechanisms have been purposely excluded to avoid making the figure overly complex.

3.1. Heart Rate and Stroke Volume

3.1.1. AUTONOMIC NERVOUS SYSTEM

Laurent et al. (1983), Taylor et al. (2007), Nilsson (2011), and Sandblom and Axelsson (2005) provide extensive descriptions on the anatomy of the autonomic nervous system and its role in affecting cardiovascular function in fishes, including elasmobranchs. Only a brief summary will be given here. Elasmobranchs are the first vertebrates to have an autonomic nervous system both anatomically and functionally separable into parasympathetic and sympathetic components (Taylor, 1992; Taylor et al., 2007; Fig. 1.6).

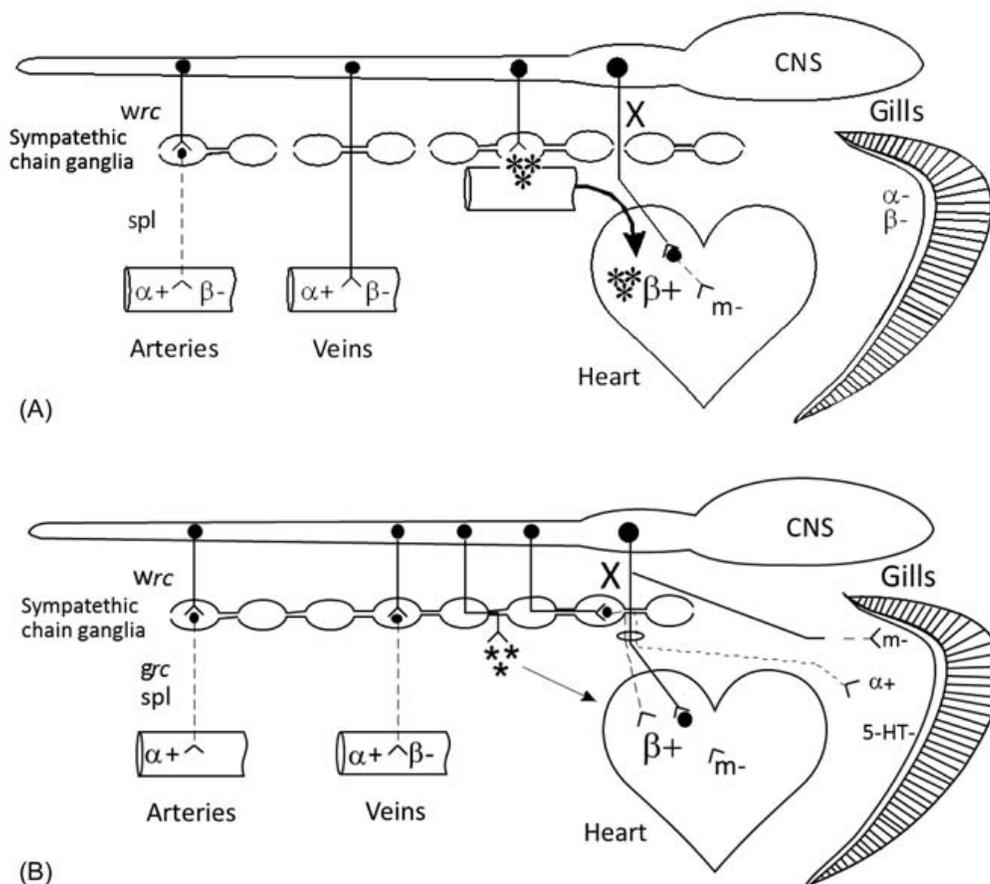


Figure 1.6. Schematic diagram of the autonomic system innervation of the heart, systemic and branchial vessel in elasmobranch (A) and teleost (B) fishes. Solid lines show preganglionic neurons, dashed lines post-ganglionic neurons. CNS, central nervous system; spl, splanchnic nerve; wrc, white rami communicans; X, vagus (or 10th cranial) nerve; +, vasoconstrictor or positive chronotropic response; -, vasodilator or negative chronotropic response; α , alpha adrenergic receptor; β , beta adrenergic receptor; *, chromaffin cells. Figure is from Sandblom, and Axelsson (2011), reproduced by permission of M. Axelsson, Department of Zoology, Göteborg University, Box 463, S-405 30 Gothenburg, Sweden.

Anatomical, histological, physiological, and pharmacological studies all indicate that sympathetic innervation is absent from elasmobranch hearts (e.g., Burnstock, 1969; Laurent et al., 1983; Nilsson, 2011; Sandblom and Axelsson, 2011; Fig. 1.6). The major control of the elasmobranch heart appears to be by variation in the activity of parasympathetic portion of the tenth (X) cranial (i.e., vagus) nerve (Short et al., 1977, 1979; Taylor and Butler, 1982; Taylor, 1992). The vagal innervation of the heart is inhibitory (i.e., activity in the vagus nerve has a negative chronotropic effect) and is antagonized by atropine (e.g., Short et al., 1977; Agnisola et al., 2003). This suggests that vagal innervation of elasmobranch hearts is cholinergic, as it is in teleosts and other vertebrates (Nilsson, 1983, 2011; Sandblom and Axelsson, 2011). There is a resting vagal tone that increases during hypoxia; and vagal nerve activity plays a role in cardio-respiratory synchrony during normoxia (Taylor and Butler, 1982; Taylor et al., 2007, 2009).

Circulating catecholamines (up to $\sim 10^{-9}$ M to $\sim 10^{-7}$ M) effectively compensate for the lack of cardiac sympathetic innervation (Butler et al., 1978; Short et al., 1977; Van Vliet et al., 1988). Both adrenaline and noradrenaline produce positive inotropic and chronotropic responses via β -adrenoreceptors in intact elasmobranch hearts and isolated myocardial tissue (Capra and Satchell, 1977b; Maylie and Morad, 1979; Van Vliet et al., 1988), although only the former appears to do so at physiologically relevant concentrations (Van Vliet et al., 1988). Humoral stimulation could take place either via catecholamines released into the anterior venous system from chromaffin tissue (Abrahamsson, 1979; Laurent et al., 1983), or by catecholamines released from specialized endothelial cells in the sinus venosus (Sætersdal et al., 1975). Both activity and acute hypoxia result in a ~ 10 x increase in the plasma concentration of circulating catecholamines (Butler et al., 1978, 1986; Randall and Perry, 1992). There also appears to be an interaction of cholinergic and adrenergic stimuli. In *in situ* perfused heart experiments, a 10^{-8} M concentration of noradrenaline in the perfusate (approximating that seen in routinely active sharks in normoxia) increases the bradycardia response to vagal stimulation, whereas 10^{-7} M noradrenaline concentration (approximating that seen in sharks acutely exposed to hypoxia) decreases it (Agnisola et al., 2003).

In contrast to heart rate and contractility, the main determinant of stroke volume in both atrial and ventricular chambers of elasmobranch hearts is end diastolic volume. Once again this topic has been extensively reviewed elsewhere (Farrell and Jones, 1992; Olson and Farrell, 2006), so only a brief summary will be presented. The two primary factors effecting atrial and ventricular end diastolic volumes are filling time (i.e., the reciprocal of heart rate) and the central venous pressure. Ventricular end diastolic volume is also obviously a function of atrial ejection volume, although the importance

of atrial contraction to ventricular filling appears to be species-specific in elasmobranchs (Lai et al., 2004). The relationship between end diastolic volume and stroke volume is characterized by the well-known positive relationship between the energy of contraction and the length of the muscle fiber known as the Frank–Starling mechanism. The end result is that both atrial and ventricular chambers tend to eject a near constant fraction of their end diastolic volumes, such that stroke volume increases in conjunction with end diastolic volume. Therefore, cardiac output in elasmobranchs tends to remain constant with decreases in heart rate, as it does in teleosts, because bradycardia occurring in response to direct vagal stimulation or hypoxia is accompanied by increases in stroke volume (Short et al., 1977; Farrell and Jones, 1992).

The Frank-Starling mechanism is an intrinsic property of the cardiac muscle itself, but in elasmobranch hearts adrenergic stimuli may also be involved. Treatment of fish with the adrenergic β -receptor antagonist propranolol abolishes the rise in stroke volume associated with hypoxia-induced bradycardia in catsharks, but it is unclear whether the catecholamines involved are circulating or locally released (Short et al., 1977). Supportive of the latter is the observation that the β -adrenergic receptor antagonist Nadolol reduces heart rate in *in situ* perfused heart preparations in spiny dogfish, implying that the source of the adrenergic tone maintaining heart rate is the heart itself (Agnisola et al., 2003). Sætersdal et al. (1975) demonstrated the existence of catecholamine-storing cell in elasmobranch hearts, and both Meghji and Burnstock (1984) and Thompson and O’Shea (1997) reported strong positive inotropic responses ($\sim 400\%$ increase in force in the latter) to applied acetylcholine in isolated ventricular strips from catshark and Port Jackson shark. These observations, in turn, imply the existence of a cholinergic-adreno complex (i.e., the applied acetylcholine has a paracrine effect causing the release of catecholamines from storage sites within the heart itself). This, in turn, implies the existence of cholinergic nerves in elasmobranch hearts terminating in proximity to catecholamine storage sites. Such a system would allow more rapid and discrete control of cardiac function than just the circulatory system-wide increase in plasma catecholamine levels resulting from stimulation of the auxiliary bodies located near the posterior cardinal sinus (Fig. 1.6; Gannon et al., 1972; Abrahamsson, 1979). Adrenergic systems have been described in mammalian hearts (e.g., Hoffmann et al., 1945; Ellison and Hibbs, 1974; Huang et al., 1996), implying that release of intrinsically produced catecholamines could be a widespread mechanism influencing myocardial function, and one that evolved in early in the vertebrate lineage.

Arterial pressure is a function of cardiac output and arterial resistance. Arterial pressure can influence stroke volume (Fig. 1.5), as it is a prime determinant of intraventricular pressure (i.e., afterload), and therefore the ventricular wall tension that must be generated during the ejection phase of

the cardiac cycle (Farrell and Jones, 1992). Although it is a prime determinant of cardiac power demand, several studies employing *in situ* isolated heart preparations have shown that arterial pressure actually has little effect on stroke volume over the physiological pressure ranges of elasmobranchs and teleosts (Davie and Farrell, 1991a; Farrell, 1991).

3.1.2. VENOUS COMPLIANCE, VENOUS TONE, AND VENOUS PRESSURE

In contrast to arterial pressure, venous filling pressure (also called central venous pressure) is a prime determinant of stroke volume (and therefore cardiac output) in that it is a prime determinant (along with heart rate or its reciprocal, filling time) of end diastolic volume (Rothe, 1986; Pang, 2001; Tyberg, 2002). Venous pressure is, in turn, primarily determined by blood volume, venous tone, and venous compliance (Olson, 1992; Olson and Farrell, 2006). The control and function of the venous circulation have received relatively scant consideration in elasmobranchs, although this aspect of the cardiovascular system has received considerable attention in teleosts (reviewed by Olson and Farrell, 2006; Sandblom and Axelsson, 2007; Olson, 2011a).

In elasmobranchs, the rate of venous blood flowing back to the heart during diastole (i.e., venous return) is a complex function of both *Vis-a-tergo* (literally “from behind”) and *Vis-a-fronte* (literally “from the front”) forces (Farrell, 2001). *Vis-a-tergo* forces result from (i) the momentum remaining in the venous blood imparted during ventricular ejection, and (ii) the pressure driving gradient from the venous system to the heart (i.e., venous pressure). The latter can be augmented by the *Vis-a-fronte* force, which is a negative pressure (i.e., sub-ambient aspirational pressure) around the sinus venosus, atria and ventricle, created by ventricular contraction and blood ejection occurring within the rigid pericardial chamber of elasmobranchs. Although *Vis-a-tergo* and *Vis-a-fronte* forces are interrelated, we will discuss them separately.

Vis-a-tergo force is venous filling pressure (also called “central venous pressure”) which is usually measured in the ductus of Cuvier in elasmobranchs (e.g., Sandblom et al., 2006, 2009). Venous filling pressure is, in turn, influenced by blood volume and vascular capacitance (Fig. 1.7; Olson, 2011a). Vascular capacitance is the relationship between blood volume and mean circulatory filling pressure, with the latter being defined as the stable blood pressure measured during brief stoppages of cardiac output (Fig. 1.7; Sandblom and Axelsson, 2006; Sandblom et al., 2006, 2009; Olson, 2011a). Because veins are substantially more distensible than arteries, vascular capacitance is primarily determined by the functional characteristics of the venous system (Olson and Farrell, 2006).

Assuming a stable blood volume, changes in mean circulatory filling pressure reflect changes in venous vascular capacitance (Fig. 1.7; Pang, 2000). Venous compliance is calculated as the change in venous blood

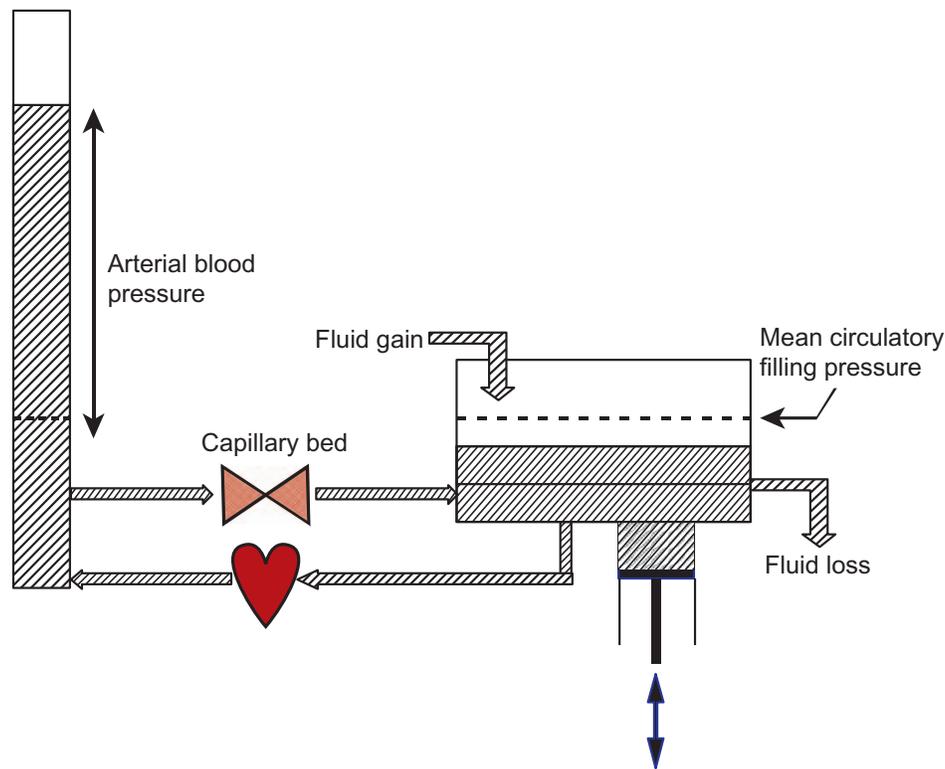


Figure 1.7. A hydraulic model of the elasmobranch circulation. The heart is a pump moving blood from the low pressure – high compliance venous circulation to the low compliance – relatively high pressure arterial circulation. Blood returns from the latter to the former through the systemic capillary bed, which in turn affects arterial blood pressure. Venous return to the heart is effected venous pressure, which is influenced by venous capacitance (represented by a piston mechanism) and blood volume of the venous system. Blood volume of the venous systems is, in turn, affected by the net difference between the rates of fluid gain and loss. The system would reach mean circulatory filling pressure in the absence of cardiac output.

volume per unit change in venous pressure (i.e., $\Delta V \cdot \Delta P^{-1}$) and represents the slope of the venous capacitance curve at any given blood volume (Olson, 2011a). More informally, venous compliance is a measure of the “stiffness” of the venous vascular system. Because compliance is the ratio of blood pressure and volume, the degree of vascular constriction (i.e., vascular tone) in the veins will be reflected in changes in venous compliance and capacitance. Depending on the circumstance, changes in venous pressure will influence venous volume, and vice versa. For example, decreases in venous compliance due to an increase in venous vascular tone can result in the shunting of blood volume from the venous system into the arterial system. This results in an increase in SV and cardiac output. For this reason, venous compliance is regarded major factor influencing SV and thus cardiac output.

Although data on processes and mechanisms affecting venous pressure and venous capacitance in elasmobranchs are limited, increases in the former have been recorded in spiny dogfish in response to adrenaline injection, and in leopard shark in response to exercise (Capra and Satchell, 1977a; Lai et al., 1989). The adrenaline response most likely reflects an increase in venous tone and a resultant decrease in venous capacitance. The latter would serve to increase venous return (Fig. 1.7), stroke volume, and cardiac output; and therefore to increase the supply of oxygen and metabolic substrates to the working muscles. More recent studies measuring changes in mean circulatory filling pressure (which is reflective of changes in vascular capacitance, Fig. 1.7) and venous tone in spiny dogfish in response to adrenergic stimulation and adrenergic receptor blockade show the presence of α -adrenergic (constrictive), β -adrenergic (dilatory) responses (Sandblom et al., 2006); and demonstrate the importance of venous capacitance to circulatory system function in elasmobranchs equivalent to the situation in other vertebrates (e.g., Rothe, 1986; Pang, 2000; Tyberg, 2002; Sandblom et al., 2005). It should be noted, however, that the venous vasculature in spiny dogfish does not appear to respond to stimulation of the baroreceptors resulting from transient stoppage of cardiac output (Sandblom et al., 2006), as it does in rainbow trout (Sandblom and Axelsson, 2005). This implies that direct control of the venous vasculature is lacking in the former but present in the latter, similar to the situation with respect to control of arterial circulation in elasmobranchs (Fig. 1.6).

We also note that hepatic vein sphincters have been described in the spiny dogfish, Pacific skate (*Raja binoculata*), and basking shark (*Cetorhinus maximus*), which constrict in response to acetylcholine and appear to control venous return from the liver (Johansen and Hanson, 1967). Relaxation of these hepatic vein sphincters could thus be a major short-term mechanism for controlling venous return of blood from the hepatic sinusoidal capillaries and thus cardiac output in elasmobranchs, especially given their large livers and the importance of *Vis-a-fronte* forces (discussed in detail below). Sphincters have also been reported in the hepatic veins of mammals, implying that similar mechanism for controlling venous return from the liver may actually be wide spread in vertebrates (Pang, 2000).

3.1.3. CARDIAC FILLING AND THE ROLE OF THE PERICARDIOPERITONEAL CANAL

We address this issue in some detail not only because of its important effect on cardiac output in elasmobranchs, but also because of relatively recent changes in our understanding of this aspect of the cardiac cycle. In all elasmobranchs, the four serially connected contractile chambers of the heart (sinus venosus, atrium, ventricle, and conus arteriosus), along with a short

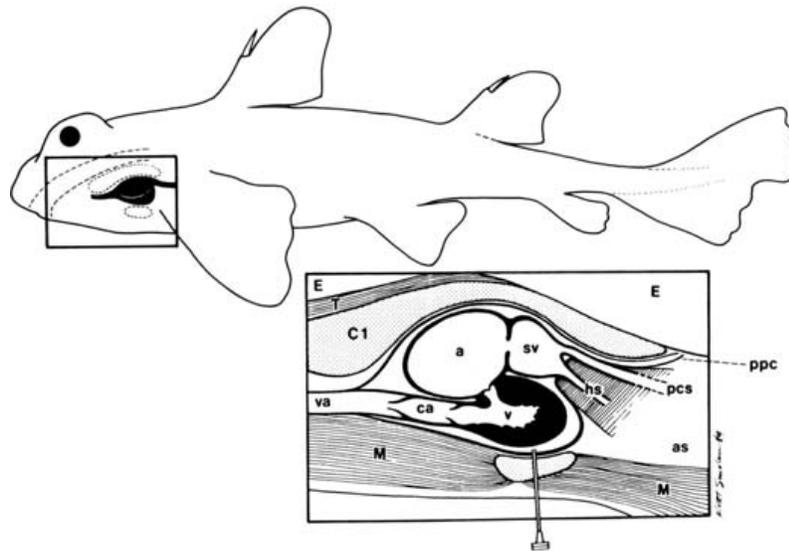


Figure 1.8. Schematic diagram showing the relative positions of heart, pericardium, and pericardioperitoneal canal (PPC) in a horn shark. The heart is surrounded by the pericardium, which is in close contact with muscle (M) ventrally and cartilage (C1) dorsally, and the anterior half of latter lies immediately below the tongue (T). The anterior limit of the pericardial space closes around the conus arteriosus (ca) and ventral aorta (va); the posterior limit is the transverse septum, which separates the pericardial space from the abdominal space (as). Blood enters from the hepatic sinus (hs) and posterior cardinal sinus (pcs) into the sinus venosus (sv), to the atrium (a), and then to the ventricle (v), conus arteriosus, and ventral aorta. The pericardioperitoneal canal opens on the ventral aspect of the esophagus (E). A cannula shown passing through the coracoid cartilage permits manipulation of pericardial fluid volume and measurement of pressure. Figure is from [Abel et al. \(1994\)](#), reproduced by permission of D. Able, Department of Marine Science, Coastal Carolina University, Conway, South Carolina.

segment of the ventral aorta, are suspended within a structurally rigid pericardial chamber – a remnant of the anterior-ventral coelom that late in embryonic development becomes separated from visceral cavity by the rise of the transverse septum ([Romer, 1971](#)). The somewhat conically-shaped pericardial chamber ([Fig. 1.8](#)), located near the floor of the pharyngeal-branchial region with its apex lying just below the oral-buccal cavity, is held up by attachments to the surrounding skeletal and muscular elements. The ventro-lateral branchial muscles and the dorsally positioned basibranchial plate provide the main bulk of support along its length. The lateral walls of the pericardial chamber are reinforced by the broad projection of the pectoral girdle, while the coracoid bar strengthens it ventrally along with the continuum of the oro-buccal somatic muscles from above ([Shabetai et al., 1985](#)).

[Schoenlein and Willem \(1894\)](#) were the first to propose a model for cardiac filling in elasmobranchs where negative pericardial pressure

(made possible by the rigid pericardium described above) has a critical role. This idea gained general acceptance (e.g., [Butler and Metcalfe, 1988](#); [Satchell, 1971, 1991](#); [Franklin and Davie, 1993](#)) following the documentation of negative pericardial pressures in elasmobranchs (e.g., [Johansen, 1965](#); [Sudak, 1965a,b](#)). In this model, venous return and filling of the sinus venosus and atrium occur mainly by suction (i.e., *Vis-a-fronte* mechanism, [Fig. 1.5](#)) due to a persistent negative (i.e., sub-ambient) pericardial pressure, which becomes more negative during ventricular systole. The result would be a tight reciprocal interaction between ventricle ejection and atrial filling and a dependence of ventricular filling on atrial systole. In other words, the elasmobranch ventricle is filled exclusively by atrial contraction because ventricular diastolic pressure remains above atrial pressure until the onset of atrial systole.

More recent and extensive studies have, however, shown that the extreme negative pericardial pressures reported in the older literature were experimental artifacts resulting from ejection of pericardial fluid via the pericardioperitoneal canal (PPC) during the handling procedures required to emplace the requisite catheters, and that negative pericardial pressures were solely a consequence of ventricular systole ([Abel et al., 1986, 1987, 1994](#); [Lai et al., 1989](#)). The PPC is a unique anatomical feature found in hagfish, elasmobranchs, and chondrosteans (sturgeons) that connects the pericardial and peritoneal cavities ([Fig. 1.8](#)). The result has been a new conceptual model of cardiac filling mechanisms in elasmobranchs that discounts the role of negative pericardial pressure and diminishes the role of atrial contraction in ventricular filling ([Lai et al., 1996, 2004](#)). Measurements of cardinal sinus pressure in chronically instrumented and conscious leopard sharks show that resting central venous pressure is above ambient (0.2 kPa). This implies that incoming venous blood retains sufficient momentum resulting from ventricular systole (possibly augmented by venous return due to contraction of the swimming muscles during activity) to flow into the sinus venosus from the ducts of Cuvier, hepatic vein, and a few minor vessels ([Lai et al., 1990b](#)). Furthermore, echo-Doppler cardiography has shown three blood flow velocities occurring at the sino-atrial orifice within the cardiac cycle. When aligned with electrocardiogram, the first and second phases are consecutively joined and span the QRS complex and T wave (i.e., they are correlated with atrial diastole and ventricular systole) and the third phase (albeit a minor contribution) is the result of contraction of sinus venosus appearing just ahead of the onset of P wave ([Lai et al., 1990b](#)). Likewise digital angiography, echo-Doppler cardiography, and simultaneous pressure measurement in the atrium and ventricle in several shark species have clearly shown that ventricular filling is biphasic. The first phase occurs during ventricular diastole due to a positive atrial to ventricular

pressure gradient sufficient to open the atrioventricular valve, and the second occurs during atrial systole (Lai et al., 1990b, 1996, 2004).

Central to the argument that negative pericardial pressure is not the agent for cardiac filling is the presence of the PPC. The PPC opens at the median top section of the transverse septum, between the sinus venosus and the esophagus, and extends as short single or bifurcated channels along the ventral wall of the esophagus before terminating in the peritoneal cavity (Goodrich, 1918). Although its morphology and size vary among species, experimental manipulations of pericardial pressure show that this flaccid duct serves to permit one-way passage of fluid from the pericardial to the peritoneal cavity (e.g., Shabetai et al., 1985; Abel et al., 1986, 1987, 1994). Moreover, as Abel et al. (1986, 1994) and Head et al. (2001) demonstrate, chronically instrumented horn sharks (*Heterodontus francisci*) equilibrate pericardial pressure to near or above ambient within ~12 h following catheter placement, and retain it at this level for as long as 27 days. The profoundly subambient pericardial pressures reported in earlier acute studies therefore appear to have been due either to (i) fluid transfer from the pericardial to peritoneal cavities via the PPC during the manipulations required for catheter placement; or (ii) the fact that specimens were retained in air and supine. The latter allowed fluid to gravitate to the upper recesses of the pericardial cavity and to flow through the PPC into the peritoneal cavity.

Sudak (1965a) was the first to suggest that the loss of fluid via the PPC influences pericardial pressure, ventricular filling, and stroke volume. The functional role of the PPC was, however, not fully appreciated until the mid-1980s when investigators constructed pressure–volume curves by incremental infusion of saline into the pericardial cavities of several anesthetized elasmobranch species. As fluid was incrementally re-introduced into a previously evacuated pericardial cavity, pressure showed an initial steep ascent, which leveled off at ~1–2 cm H₂O, which resulted in a sigmoid-shape pressure profile (Shabetai et al., 1985, Abel et al., 1986, Head et al., 2001). This plateau was termed the “pericardial canal opening pressure”, after which further infusion of fluid drained into the peritoneal cavity. Shabetai et al. (1985) subsequently proposed three possible functions for the PPC: (i) facilitation of changes in cardiac size via displacement of pericardial fluid, (ii) lessening or prevention of cardiac compression when pericardial fluid volume increases excessively, and (iii) compensation for a poorly developed lymphatic system. Abel et al. (1986) and Head et al. (2001) have shown that when pericardial pressure is raised toward PPC opening pressure (i.e., well above ambient), or when an excessively negative pericardial pressure is applied, stroke volume in both California horn and blue sharks declines dramatically. These results suggest that the optimal operating

pericardial pressure is between slightly below to slightly above ambient, and suggest a critical role for the PPC in cardiac function.

Pericardial fluid can also be expelled through the PPC during natural processes (e.g., swimming, feeding, or evading predators), which results in a transitory drop in pressure that appears to be tolerable (Abel et al., 1986, 1994; Lai et al., 1989). This situation has not, however, been shown to result in highly negative pericardial pressure (Lai et al., 1989, 1990b). We contend, therefore, that pericardial pressure in elasmobranchs is a consequence of the interactions of cardiac stroke volume and pericardial fluid volume; the latter being influenced by fluid movements via the PPC. Experimental evidence supports these ideas. When cardiac output increases during exercise in leopard sharks, there is a near matching of increased stroke volume (0.45 ml kg^{-1}) and pericardial fluid loss (0.6 ml kg^{-1}). Moreover, pericardial pressures remain elevated well above ambient (0.09 to 0.43 kPa) further contradicting the importance of negative pericardial pressure (Lai et al., 1989). During the transitions from rest to exercise in leopard sharks, ventricular diastolic pressure increases more than pericardial pressure resulting in a higher mean ventricular diastolic transmural pressure (a measure of preload), as does cardinal sinus pressure (a measure of venous return) and contractility (Lai et al., 1990b).

3.2. Body Fluid Volume and Blood Pressure Regulation

Body fluid volume, iono- and osmo-regulation in elasmobranchs have been extensively reviewed (e.g., Pang et al., 1977; Hammerschlag, 2006; Anderson et al., 2007) and will not be extensively discussed here. Rather we limit our description to those endocrine and paracrine mechanisms most directly relevant to blood volume and blood pressure, and therefore to cardiovascular function. Since this was first described in the 1930s (Smith, 1931), there has been general consensus that marine elasmobranchs maintain body fluids from isosmotic, to slightly hyperosmotic, to seawater through the retention of 300–400 mM urea and methylamines (primarily trimethylamine oxide) (Anderson et al., 2007). Elasmobranchs thus face some passive water gain, which is excreted primarily by the kidneys (Evans et al., 2004). So unlike marine teleosts, which are hypoosmotic to the environment, elasmobranchs face a constant (albeit probably minor) problem of plasma volume expansion.

Blood and extracellular fluid volume regulation in fishes in general (including the influence of the renin–angiotensin system), the evolution of the renin-angiotensin system, and its role in volume and blood pressure regulation in elasmobranchs specifically are described in detail by Olson (1992), Nishimura (2001) and Anderson et al. (2001), respectively.

Renin, produced and subsequently released by cells associated with the kidney in response to hypotension or dehydration, cleaves angiotensinogen into a smaller decapeptide (angiotensin), which is further cleaved to the active peptides – designated as angiotensins I–IV. Due to its apparent ubiquity, the renin-angiotensin system appears to be of ancient lineage within the vertebrates (Nishimura, 2001). Its importance in body fluid volume and blood pressure regulation in teleosts is well established, but its functional role(s) in the elasmobranch cardiovascular system remains somewhat less clear. The general consensus is that the renin-angiotensin system does not play a role in regulating resting blood pressure, but it rather comes into play during hypotensive events, such as those resulting from blood loss (Bernier et al., 1999; Anderson et al., 2001). In spiny dogfish, hypotensive stimuli result in increases in circulating levels of angiotensin and simultaneous increases in plasma catecholamines (Carroll et al., 1984; Bernier et al., 1999), which suggests that the former stimulates the latter. Additionally, studies with specific blockers show that catecholamines have the dominant role in the vasoconstrictive pressor effect (Bernier et al., 1999). In contrast, studies with Japanese banded dogfish, *Triakis scyllia*, and catsharks showed (i) the presence of angiotensin receptors and the ability of angiotensin to cause constriction of isolated vessels (Tierney et al., 1997b; Hamano et al., 1998) and (ii) a direct (i.e., not catecholamine-mediated) vasoconstrictor effect of angiotensin *in vivo* (Tierney et al., 1997a). These observations suggest important differences in the responsiveness of the vascular system to angiotensin within the elasmobranch clade, and demonstrate the need for inclusion of a broader phylogenetic range of species in these types of studies.

The kallikrein-kinin system is well characterized in mammals (e.g., Bhoola et al., 1992), and has received considerable attention in nonmammalian vertebrates, including teleosts (e.g., Olson, 1992; Conlon, 1999). Similar to the renin-angiotensin system, bradykinin (the active circulating nonapeptide endproduct) in fish is produced by cleavage of an inactive precursor (kininogen) by the protease kallikrein (Wong and Takei, 2013). The cardiovascular actions of bradykinin are known in teleosts and generally involve complex multistage vasopressor-vasodepressor effects of relative short (i.e., minutes) duration (Olson, 1992; Olson and Farrell, 2006). We know of only three studies that to date have demonstrated the presence of kallikrein-kinin system in an elasmobranch (Richards et al., 1997a,b; Anderson et al., 2008). Dasiewicz et al. (2011) measured *in vivo* hypertensive and *in vitro* vasoconstrictive effects (on the mesenteric, coeliac, and first branchial artery) of homologous bradykinin in the little skate, *Leucoraja erinacea*, although the same compound caused relaxation of the ventral aorta of this species (Anderson et al., 2008). Moreover, the vasoconstrictive

response was reduced is the presence of phentolamine, an α -adrenergic agonist, implying the involvement of circulating catecholamines.

Natriuretic peptides (NPs) are a family of hormones, predominately of cardiac origin, significantly involved in sodium and volume regulation in vertebrates (Takei et al., 2007; Martinez-Rumayor et al., 2008). Multiple NPs exist in teleosts and tetrapod vertebrates, although only C-type natriuretic peptide (CNP) is found in the heart and brain of elasmobranchs (Loretz and Pollina, 2000; Takei et al., 2006; Johnson and Olson, 2008). Of the NP family, elasmobranch CNP is considered closest to the ancestral molecule and the most highly conserved in that it shares 85% identity with mammalian CNP (Loretz and Pollina, 2000; Takei et al., 2007). The functional roles of the NPs in fishes are complex and not fully understood. In elasmobranchs, NPs are plasma ion and volume reducing hormones, as they are in other vertebrates, with the major target organ being the elasmobranch rectal gland (Loretz and Pollina, 2000; Toop and Donald, 2004; Johnson and Olson, 2008). NPs also have effects on the vascular system in teleosts, and CNP specifically has been shown to cause vascular relaxation in spiny dogfish (Bjening et al., 1992; Evans et al., 1993). Because of this, and the influence of blood volume on venous return, CNP secretion in elasmobranchs could potentially reduce venous return (and therefore cardiac preload, stroke volume, and cardiac output) and arterial pressure (and therefore cardiac afterload). Overall, CNP secretion (which occurs in response to cardiac muscle stretch) could therefore serve as a potent negative feedback mechanism for reducing cardiac power when required. Indeed, Farrell and Olson (2000) have argued a cardioprotective effect is a primary function of NPs in fishes. To the best of our knowledge, the influence of CNP on venous tone and venous compliance in elasmobranchs remains unstudied, but could be a fruitful area of research to further delineate the influences of CNP release on elasmobranch cardiovascular function, including its cardioprotective effect.

We also note, however, that bolus injection of CNP into intact spiny dogfish results in an increase then decrease (i.e., bi-phasic) change in blood pressure. This includes an initial increase in vascular resistance, followed by a decrease in cardiac output that results from a decrease in stroke volume unaccompanied by a compensatory increase in heart rate (McKendry et al., 1999). Accompanying these responses is a 15-fold increase in the circulating noradrenaline level, unaccompanied by any increase in adrenaline (McKendry et al., 1999). CNP does not, however, directly stimulate catecholamine release from elasmobranch chromaffin cells (Montpetit et al., 2001); and the mechanism(s) and source(s) responsible for noradrenaline release remain unclear. Nonetheless, direct injection of noradrenaline does cause a decrease in central venous pressure in dogfish (Capra and Satchell,

1977a), which suggests that this mechanism may likewise enhance the depressor effect of CNP and thus enhance its cardioprotective effect. We speculate, however, that elasmobranch hearts may serve as a double endocrine organ possibly excreting catecholamines (described in [Section 3.1.1 Autonomic Nervous System](#)) and NPs (e.g., [Cousins and Farrell, 1996](#); [Farrell and Olson, 2000](#)). Teleosts, in contrast, appear to have lost cardiac chromaffin tissue and evolved the now well-described system of direct sympathetic innervation ([Sandblom, and Axelsson, 2011](#)).

4. SIGNALING MECHANISMS EFFECTING BLOOD VESSEL DIAMETER

In this section we briefly explore the signaling mechanisms involved in the control of vascular smooth muscle cells in elasmobranchs. The mechanisms present in vertebrates are summarized diagrammatically in [Fig. 1.9](#), with mechanisms present in tetrapod vertebrates, but not fishes, indicated in red. The control mechanisms include the pivotal role of the blood vessels themselves acting as an endocrine/paracrine/autocrine organs, as endothelial and vascular smooth muscle cells are now well known to produce several vasoactive substances (e.g., endothelins, prostaglandins, and gasotransmitters) ([Inagami et al., 1995](#); [Olson and Donald, 2009](#)). We want to emphasize, however, that changes in blood vessel diameter regulating blood flow also obviously influence blood pressure (through changes in vascular resistance) and blood volume distribution; the latter two, in turn, influence cardiac output. The mechanisms described in the following section are therefore intimately tied to the cardiovascular functional processes described in the preceding sections. The separation of this section from the previous ones is, therefore, solely for organizational convenience.

4.1. Gasotransmitters

The importance of gasotransmitters (carbon monoxide, CO; hydrogen sulfide, H₂S; and nitric oxide, NO) on blood vessel constriction and relaxation (and therefore blood flow and blood volume distribution) in vertebrates is now clearly recognized (reviewed by [Olson and Donald, 2009](#)). The first of these (CO), including its production in the endothelium and vascular smooth muscle via the catabolism of heme by heme oxygenase and its vasodilatory function, have been extensively investigated in mammals ([Wu and Wang, 2005](#)). But to the best of our knowledge the effects of CO and its role in control of blood flow in elasmobranchs remain unstudied.

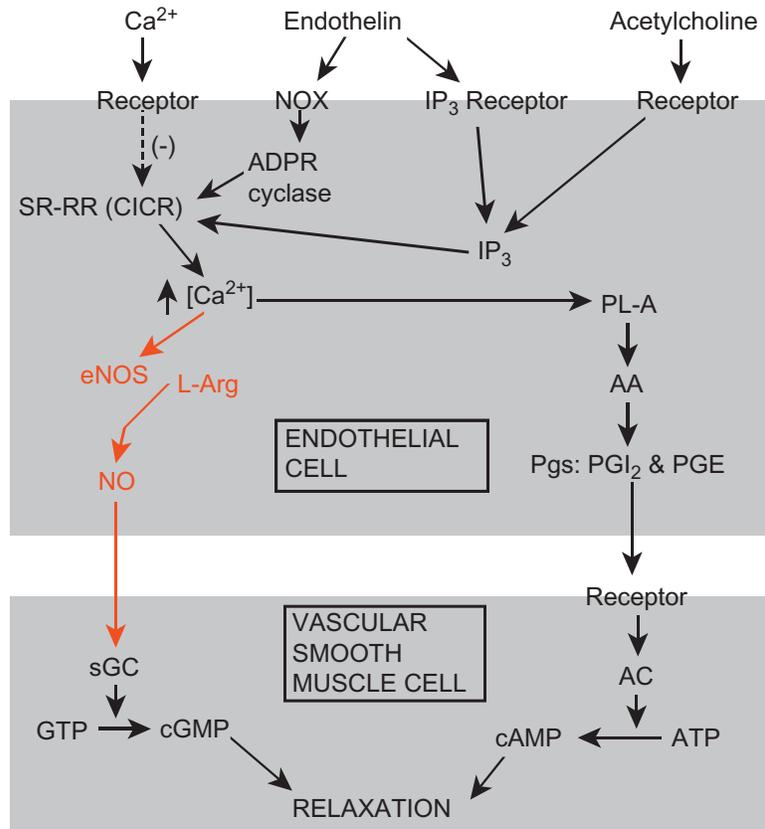


Figure 1.9. Endothelium-dependent signaling pathways controlling vascular smooth muscle cells involving nitric oxide (NO) and prostaglandin (Pgs). AA, arachidonic acid; AC, adenylyl cyclase; Ach, acetylcholine; COX, cyclooxygenase; eNOS, endothelial NOS; IP₃, inositol triphosphate; L-Arg, L-arginine; NOX, NAD(P)H oxidases; PGI₂, prostacyclin; PL-A, phospholipase A; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; SR-RR (CICR), sarcoplasmic reticulum ryanodine receptor (calcium induced calcium release). Figure is redrawn from Evans and Gunderson 1998, and includes data presented in [Evans et al. \(1996\)](#) and [Fellner and Parker \(2002, 2004, 2005\)](#).

We are aware of only one publication on the effects of CO on fish blood vessels that employed rainbow trout (*Oncorhynchus mykiss*) and lamprey (*Petromyzon marinus*) ([Dombkowski et al., 2009](#)). In both species, CO caused relaxation of vessels pre-constricted with norepinephrine, implying that this gasotransmitter mechanism is phylogenetically ancient and maybe widespread among fishes. The role of CO in modulation of blood flow in elasmobranchs is clearly in need of scrutiny.

H₂S is likewise an important vasoactive signaling molecule ([Olson, 2005, 2008, 2011b, 2013](#); [Olson and Donald, 2009](#); [Wang, 2011](#)), although it has received less attention than NO (described below). Concentration of H₂S is a

simple balance of its continuous production in the cytoplasm (from cystine) and its metabolism to inactive forms (to either sulfite or sulfate, or both) in the mitochondria (Olson et al., 2006; Mancardi et al., 2009). The difference between H₂S production and metabolism (which is oxygen-dependent) is, therefore, highly suitable as a transduction mechanism for sensing oxygen levels in a variety of tissues (Olson, 2013). Contrary to earlier reports, H₂S does not serve as blood-borne signaling molecule as sulfide is rapidly taken up by red blood cells (where it is either metabolized or bound to hemoglobin) thus reducing plasma levels to undetectable concentrations (<100 nM total sulfide, Whitfield et al., 2008). This is especially true in teleost fishes because of the presence of mitochondria in their metabolically active red blood cells (Whitfield et al., 2008). H₂S therefore functions solely as paracrine or autocrine messenger. Circulating levels of H₂S have not been measured in elasmobranchs, but we assume the situation is likewise.

Throughout the vertebrates, H₂S has two interrelated roles: (i) modification of vascular tone and therefore blood flow (through its production and destruction in vascular smooth muscle) to suit tissue metabolic needs in the face of changing oxygen levels (Wang, 2011; Forgan and Forster, 2012), and (ii) sensing either internal or external oxygen levels (Olson, 2008, 2013; Wang, 2011). The vascular effects of H₂S generally mimic those of hypoxia, in that these effects cause vasoconstriction, vasodilation, or some sequential multiphasic combination thereof (Dombkowski et al., 2005; Olson, 2005, 2013; Russell et al., 2008, Olson et al., 2006). To the best of our knowledge, however, only one elasmobranch species (sandbar shark) has been investigated with respect to the vasoactive effects of H₂S (Dombkowski et al., 2005). As opposed to the complex dose-dependent vascular responses seen in other vertebrates, H₂S produces only vasorelaxation of the dorsal aorta, ventral aorta, and afferent branchial artery and does so at a single definitive threshold concentration (Fig. 1.10; Dombkowski et al., 2005; Olson and Donald, 2009). It is therefore unknown if different vessels would have the same threshold-type response, or whether there are species-specific differences within the elasmobranchs with respect to vascular H₂S sensitivity. These are important issues as they undoubtedly relate to species-specific tolerance of ambient H₂S. Moreover, elasmobranch species' exposure to H₂S will vary widely depending on feeding mode, geographic area, season, and levels of environmental degradation (e.g., industrial pollution, nutrient runoff, environmental hypoxia, etc.) (Bagarinao, 1992). H₂S is also intimately linked with the cardiorespiratory responses of vertebrates to hypoxia through its role in oxygen sensing in neuroepithelial (putative chemoreceptor) cells of the carotid body and gills, and the chromaffin cells associated with systemic veins (Olson, 2008, 2011b, 2013). Yet these latter areas too remain unstudied in elasmobranchs, in spite

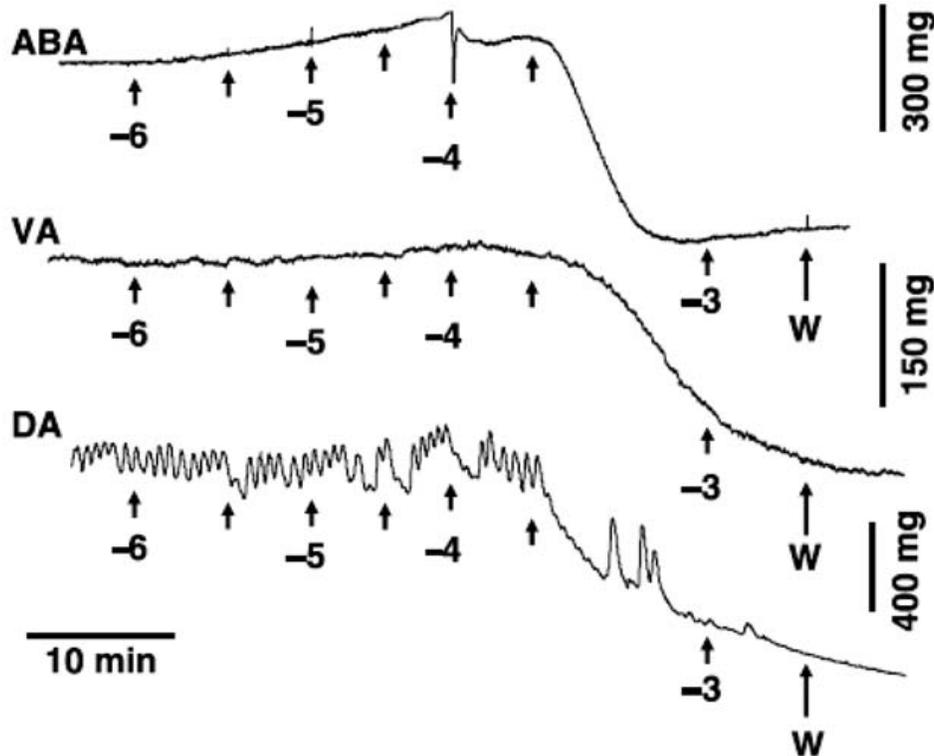


Figure 1.10. Cumulative dose-response of the afferent branchial artery (ABA), ventral aorta (VA), and dorsal aorta (DA) from sandbar shark to sodium sulfide (NaHS, which forms H_2S in solution). Values below the small vertical arrows show the log of the molar concentration of NaHS. Unlabeled arrows show the molar concentration of NaHS three times the previous value. Responses in all vessels occurred at a NaHS concentration of $\sim 3 \times 10^{-4}$ M. The “W” at the end of each trace shows the point where vessels were subjected to two consecutive washes. Figure is from [Dombkowski et al. \(2005\)](#), reproduced by permission of K. Olson, Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana.

of their importance with respect to species-specific tolerance of hypoxia, and the ecological and conservation implications thereof (discussed in [Section 2.2 Responses to Hypoxia](#)).

The pivotal role of NO as a vasoactive signaling molecule in mammalian blood vessels was recognized several decades ago (reviewed by [Furchgott, 1999](#)) and the mechanisms of production and the physiological actions of NO in mammalian and nonmammalian vertebrates has subsequently been extensively and intensively investigated (reviewed by [Umans and Levi, 1995](#); [Nilsson and Söderström, 1997](#); [Olson and Donald, 2009](#)). In brief, NO is produced from L-arginine by nitric oxide synthase (NOS), which (in tetrapod vertebrates) exists in three isoforms: (i) brain or neuronal NOS (nNOS) expressed in the parasympathetic postganglionic perivascular

nitroergic nerves, (ii) an inducible form (iNOS) requiring DNA transcription and protein synthesis, and (iii) endothelial NOS (eNOS) expressed by the endothelial cells (Griffith and Stuehr, 1995; Ricciardolo et al., 2004; Olson and Donald, 2009).

The presence of eNOS has been difficult to document definitively in fishes and remains in dispute (this pathway of NOS production is therefore shown in red in Fig. 1.9). In contrast, the nNOS isoform is clearly present in teleost fishes (Fritsche et al., 2000; Donald and Broughton, 2005; Hyndman et al., 2006; Olson and Donald, 2009; González-Domenech and Muñoz-Chápuli, 2010). In addition, there is histochemical evidence that major blood vessels (including arteries and veins) of both the giant shovelnose ray and epaulette shark are innervated by nitroergic nerves (i.e., those expressing nNOS), as are the vessels of the gut in spiny dogfish (Olsson and Holmgren, 1997; Donald et al., 2004; Donald and Broughton, 2005). Hypoxia is well known to up-regulate iNOS gene expression in mammals (Ricciardolo et al., 2004), and appears to do so likewise in the vasculature of the brain in the epaulette shark (Renshaw and Dyson, 1999). Assuming the NO produced causes vasodilation, this mechanism could help maintain oxygen delivery and neural function. Indeed, hypoxic vasodilation (measured as a drop in blood pressure in intact animals) appears to result from up-regulation of iNOS and increased circulating levels of NO in spiny dogfish (Swenson et al., 2005).

In teleost fishes, NO or NO donors generally cause marked relaxation of vascular smooth muscle (e.g., Jennings et al., 2004), although NO has also been reported to cause vasoconstriction of the branch circulation (Pellegrino et al., 2002). But in contrast to teleosts, the direct role of NO in control of blood vessel diameter in elasmobranchs has been difficult to document (e.g., Donald et al., 2004). Several studies have found no vasodilation of isolated elasmobranch blood vessels in the presence of a NO donor *in vitro* (Evans and Gunderson, 1998a; Evans, 2001; Donald and Broughton, 2005) suggesting that elasmobranchs lack the NO signaling pathway in the smooth muscle present in teleosts. The functional role(s) of the nitroergic nerves in elasmobranchs described above therefore remains largely unresolved.

4.2. Endothelins and Prostaglandins (Prostacyclin)

Endothelins are a family of peptides containing 21 amino acids, encoded by three separate genes designed ET-1, ET-2, and ET-3. Endothelins are involved in control of blood flow, blood pressure, systemic vascular resistance, and venous capacitance in vertebrates. They are produced predominately (but not exclusively) by endothelial cells (Fig. 1.9) and exert a local paracrine function (Kohan et al., 2011). Endothelins are well

recognized as potent vasoconstrictors in teleost and elasmobranch fishes (e.g., [Poder et al., 1991](#); [Olson et al., 1991](#); [Evans et al., 1996](#); [Hoagland et al., 2000](#); [Evans, 2001](#); [Perry et al., 2001](#); [Stenslkken et al., 2006](#)). To the best of our knowledge, the proximal stimuli for release of endothelins in fishes is undetermined, although in mammalian vascular tissue mechanical stretch and increases in intracellular Ca^{2+} both result in endothelin release ([Russell and Davenport, 1999](#); [Kohn et al., 2011](#)). [Evans and Gunderson \(1998a\)](#) have demonstrated that the relaxation of the ventral aorta from smooth dogfish in response to acetylcholine results from the production of a prostaglandin, not NO ([Fig. 1.9](#)). This pathway, however, involves release of Ca^{2+} from sarcoplasmic reticulum–ryanodine receptor (SR–RR) of the endothelial cell via the participation of inositol triphosphate (IP₃) ([Evans and Gunderson, 1998a](#)). Because the increase in intracellular Ca^{2+} does not apparently accompany release of endothelins from the endothelial cell (otherwise the vasodilation of the vascular smooth muscle in response to prostaglandin would presumably have been overridden), we have therefore drawn this pathway for production of endothelins in red ([Fig. 1.9](#)), as the current preponderance of evidence is against its existence in elasmobranchs.

Endothelin receptors are classified into ET_A and ET_B subtypes. Stimulation of the former produces purely vasoconstrictive responses, whereas stimulation of the latter can produce vasoconstriction or vasodilation, with vasodilation through the stimulation of NO or prostaglandin production by the endothelial cell ([Fig. 1.9](#); [Fellner and Parker, 2004](#); [Kohan et al., 2011](#)). Based on binding studies, it appears that only ET_B receptors are present in elasmobranchs ([Evans and Gunderson, 1999](#); [Fellner and Parker, 2004](#)). In those tissues where endothelins cause vasodilation, we presume the response is due to the stimulation of production of prostaglandins by the endothelial cell, as described above for acetylcholine ([Fig. 1.9](#)). The pathway for endothelins causing vasoconstriction in the vascular smooth muscle cell in elasmobranchs is the production of IP₃, activation of the IP₃ and ryanodine receptors on the sarcoplasmic reticulum (SR), and the release of Ca^{2+} from SR which is subsequently reinforced through Ca^{2+} induced Ca^{2+} release ([Fig. 1.9](#); [Fellner and Parker, 2005](#)).

The subcellular mechanisms whereby endothelins affect vascular smooth muscle are understood in elasmobranchs ([Fellner and Parker, 2004, 2005](#)), as are their specific effects in various organ systems. Exogenously applied endothelins cause vasoconstriction in a variety of systemic blood vessels (e.g., [Olson et al., 1991](#); [Evans et al., 1996](#); [Evans, 2001](#)) and induce contraction of pillar cells in the gill lamella. Endothelins thereby affect ventral and dorsal aortic (i.e., arterial) blood pressure, gill vascular resistance, and gas exchange ([Hoagland et al., 2000](#); [Perry et al., 2001](#);

Stensløykken et al., 2006). The influence of endothelins on elasmobranch myocardium remain unstudied, although they do have a positive inotropic affect in teleost hearts and increase CNP secretion (Vierimaa et al., 2006). Their role in maintaining overall physiological homeostasis in response to changing environmental conditions in fishes remains unclear, however.

4.3. Autonomic Nervous System Signaling Mechanisms (Adrenaline and Noradrenaline)

The anatomy of the autonomic nervous system in elasmobranchs and its role in controlling the vasculature has been described in detail elsewhere (e.g., Nilsson, 1997; Nilsson and Holmgren, 1988; Tota and Cerra, 2009; Sandblom and Axelsson, 2011) so only a brief summary is given here. The autonomic nervous system and its control of the vasculature are less developed in elasmobranch than in teleost fishes (Fig. 1.6). Control of vasculature by the sympathetic portion of the autonomic nervous system is considered to be primarily by circulating catecholamines derived from paravertebral autonomic ganglia (“chromaffin tissue” or “auxillary bodies”) associated with the post cardinal vein (Holcombe et al., 1980; Bernier et al., 1999; Satchell, 1999; Perry and Capaldo, 2011; Sandblom and Axelsson, 2011). However, the presence of sympathetic nerve ending in the walls of major blood vessels has been demonstrated through fluorescent histochemistry in spiny dogfish (Nilsson and Holmgren, 1988). Thus the blood vessels in the gut also appear to receive some direct sympathetic innervation (Nilsson et al., 1975; Holmgren and Nilsson, 1983). Various parts of the vasculature also show cholinergic responses (Farrell and Johansen, 1995; Evans and Gunderson, 1998b), although neither the systemic nor branchial blood vessels receive direct parasympathetic cholinergic innervation (Donald, 1998). Rather acetylcholine may “spillover” from the parasympathetic cholinergic nerves (vagus or tenth cranial nerve) innervating the heart, (Nilsson, 1983; Metcalfe and Butler, 1984; Evans and Gunderson, 1998b), but the exact source of the transmitter (acetylcholine) remains unresolved.

The vasoconstriction, vasodilation, or biphasic responses observed in isolated coronary, pre- and postbranchial arteries exposed to catecholamines or specific α - and β -adrenergic agonists are explained by the presence of both α - and β -adrenergic receptors in elasmobranch systemic and coronary blood vessels (Capra and Satchell, 1974a,b; Bennett, 1993, 1996; Farrell and Davie, 1991a,b). The various organ and tissue-level responses observed *in vivo* (e.g., Capra and Satchell, 1974a; Holmgren et al., 1992) must therefore depend primarily on the relative numbers of α - and β -receptors, their affinities, and their anatomical locations rather than different innervation patterns in organ- or tissue-level blood vessels. Spiny

dogfish have been shown to maintain significant resting adrenergic arterial vasomotor tone (Holcombe et al., 1980). Arguably, however, the primary function of the increase in circulating catecholamines produced by the adrenergic nervous system is to allow animals to survive stressful conditions (Perry and Capaldo, 2011). Stressful conditions (e.g., severe hypoxia, hypoxemia, exhaustive exercise, hypotension) which cause catecholamine secretion in lower vertebrates (including elasmobranchs), and the proximate neuroendocrine mechanisms involved are described in detail by Reid et al. (1998) and Perry and Capaldo (2011). In this regard teleosts and elasmobranchs appear to be largely similar. The primary proximal trigger mechanism is the release of acetylcholine by preganglionic sympathetic neurons, which results in membrane depolarization, Ca^{2+} entry and subsequent release of catecholamines from the adrenergic neurons (Reid et al., 1998). A variety of noncholinergic mechanisms/transmitters have been identified as being involved in catecholamine release from chromaffin tissue. These include an increase in plasma potassium levels (resulting in membrane depolarization and catecholamine release), Ca^{2+} entry into adrenergic nerve endings, peptidergic neurotransmitters, the gasotransmitters (NO, H_2S), histamine, and various hormonal signals including angiotensin (Opdyke et al., 1983; Reid et al., 1995, 1998, Bernier et al., 1999).

4.4. Other Vascular Signaling Mechanisms (Acetylcholine, Adenosine, CNP, Serotonin, Vasoactive Intestinal Polypeptide, Bombesin, and Neuropeptide Y)

Like catecholamines, acetylcholine has significant vasomotor effects in fishes although (as described above) its source and overall role *in vivo* are not well understood. Specifically, in contrast to mammals, acetylcholine causes constriction of systemic vessels even in the absence of an intact endothelium in teleosts and elasmobranchs through stimulation of receptors on the smooth muscle cell (Fig. 1.9; Evans and Cegelis, 1994; Evans and Gunderson, 1995, 1998b; Farrell and Johansen, 1995; Evans, 2001). In contrast, acetylcholine produces vasodilation in coronary blood vessels (at concentrations about 0.1 mM) in rough skate (*Raja nasuta*) and mako shark (Farrell and Davie, 1991a,b), presumably through the prostaglandin mechanism shown in Fig. 1.9 (Evans et al., 1996). These contrasting effects show the presence of vessel specific responses and speak to the complexity of control of the vascular system in elasmobranchs, which remains poorly understood at the organ and whole animal level (as opposed to the cellular and subcellular level), as well as species-specific differences and how these relate to the ability of elasmobranchs to tolerate various environmental conditions, recover from exhaustive exercise, meet metabolic demands, etc.

Indeed, very few studies have looked at factors affecting vasoactivity across a range of elasmobranch species. One of the few that has (Olson et al., 2000) found the spontaneous contractions (characteristic of elasmobranch but not teleost vessels) to be vessel- and species-specific, with a correlation between a species' activity pattern (i.e., sedentary vs. active) and a propensity for spontaneous contractions. We contend that these observations again demonstrate the need for investigations involving representatives from a broader range of elasmobranch phylogenetic groups or ecomorphotypes.

The roles of adenosine in control of vascular and cellular function are well characterized in tetrapod vertebrates (e.g., Shryock and Belardinelli, 1997; Tabrizchi and Bedi, 2001). Adenosine is released by cells under stressful conditions, such as a mismatch between metabolic oxygen demand and supply. In mammalian vessels, adenosine produces either relaxation or contraction depending on vessel tone (Tabrizchi and Bedi, 2001). In the few elasmobranch species where this has been investigated, adenosine (or adenosine agonists) causes either: (i) only vasoconstriction, (ii) vasoconstriction at low concentrations and vasodilation at higher concentrations (Farrell and Davie, 1991a,b; Evans, 1992), or (iii) only vasodilation (Söderström et al., 1999). A more systematic study of the role of adenosine across a range of blood vessel types, and involving a larger number of elasmobranch species, is clearly warranted.

As mentioned previously, C-type natriuretic peptide (primarily produced by the heart in elasmobranchs, Schofield et al., 1991; Suzuki et al., 1991) is involved in blood pressure regulation. Several studies using isolated endothelium-free vascular tissue have shown C-type natriuretic peptide to be a potent vasodilator (Evans, 1991; Evans et al., 1993), functioning presumably through the particulate guanylate cyclase-linked (pGC) receptors. Stimulation of these receptors results in production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) within the smooth muscle cell (Fig. 1.9), as occurs in shark rectal gland tissue (Gunning et al., 1993, 1997; Aller et al., 1999; Silva et al., 1999; Scotland et al., 2005).

The nonadrenergic noncholinergic (NANC) nerves (i.e., those releasing serotonin, vasoactive intestinal polypeptide, bombesin, and neuropeptide Y) in fishes are described in detail by Nilsson and Holmgren (1992) and only a brief description will be given here. In general, the functional roles of the transmitters and the stimuli causing their release remain unclear in elasmobranchs. Serotonin (5-hydroxytryptamine) containing nerves have been identified surrounding gut blood vessels (Tagliaferro et al., 1988), and the compound does cause small increases in ventral aortic blood pressure *in vivo* (Reite, 1969). Nerve fibers containing vasoactive intestinal polypeptide have been demonstrated in the walls of the coeliac and mesenteric

arteries of spiny dogfish (Holmgren and Nilsson, 1983). This neurotransmitter does have significant vascular effects in mammals and teleosts. Nevertheless it has been shown to cause vasoconstriction in some elasmobranch blood vessels, but also vasodilation and increased glandular secretion in shark rectal gland (Thorndyke et al., 1989; Nilsson and Holmgren, 1992). Its overall role in the cardiovascular system of elasmobranchs remains unclear. Bombesin, like vasoactive intestinal polypeptide, has been located in nerves associated with blood vessels of the gut (in spiny dogfish) and the coronary artery (in longnose skate, *Raja rhina*) (Bjenning et al., 1990, 1991; Holmgren and Nilsson, 1983). Bombesin shows vasoactivity, causing vasodilation in arteries of the stomach and vasoconstriction of isolated coronary arteries, which suggests that it may be involved in blood flow redistribution (Bjenning et al., 1990, 1991; Nilsson and Holmgren, 1992). Neuropeptide Y belongs to a family of regulatory peptides that can be isolated from the pancreas and brain of vertebrates, including teleost and elasmobranch fishes (e.g., Vallarino et al., 1988; Conlon et al., 1991b, 1992; Bjenning et al., 1993). It is arguably the best studied of the NANC neurotransmitters, although its exact role in cardiovascular function in elasmobranchs also remains unclear. Neuropeptide-Y containing fibers have been located in nerves associated with blood vessels of the gastrointestinal system, ventricle, and conus arteriosus (Conlon et al., 1991a; Bjenning et al., 1993). The neurotransmitter has been shown to cause vasoconstriction in vessels denuded of the endothelium, indicating that it has a direct effect on vascular smooth muscle cells (Bjenning et al., 1993), and to have a positive chronotropic effect on isolated perfused spiny dogfish hearts (Xiang et al., 1994).

4.5. Substances Affecting Gill Blood Flow Patterns

As is evident from reviews of gill anatomy, function, blood flow patterns, and the mechanism controlling them (Olson, 2002a,b; Sundin and Nilsson, 2002; Evans et al., 2005; Jonz and Zaccane, 2009), the literature on elasmobranchs is relatively sparse compared to the number of studies on teleosts. We will therefore restrict our description to mechanisms that have been demonstrated in elasmobranch gills, although we suspect other endocrine, paracrine, or autocrine mechanisms (equivalent to those of teleost gills) are present but remain to be described. As shown in Fig. 1.6, in contrast to teleosts, the branchial vasculatures of elasmobranchs are generally considered not to receive direct autonomic innervation (e.g., Taylor et al., 1999, 2009); rather vasomotor control is by circulating catecholamines, hormones, or local paracrine factors. Gill vasculatures in spiny dogfish and blacktip reef shark have been shown to possess both α

(constrictor) and β (dilatory) receptors, although the latter appear to dominate (Evans and Clairborne, 1983; Metcalfe and Butler, 1984; Chopin and Bennett, 1995). Likewise, the efferent branchial arteries from the lemon and blacktip reef sharks have been shown to have similar α - and β -adrenergic responses, which implies that these vessels also play a role in controlling blood pressure drop across the gills, and therefore affect gill blood flow patterns (Bennett, 1993, 1996). Acetylcholine increases vascular resistance in catsharks (Davies and Rankin, 1973; Metcalfe and Butler, 1984) and stingaree species (*Urolophus mucosus* and *U. paucimaculatus*) (Donald, 1988); however, as with the systemic vasculature, its source *in vivo* remains unclear. The mechanism of action of acetylcholine is presumably directly on the smooth muscle cells (Fig. 1.9) as it is in teleost gills (i.e., it is independent of the presence of an intact endothelium, Pellegrino et al., 2002). Natriuretic peptides are branchial vasodilators in teleosts (Olson, 2002b) and natriuretic peptide receptors have been demonstrated in the gills of spiny dogfish (Donald et al., 1997), so we assume this family of hormones produces similar responses in elasmobranch gills, although to the best of our knowledge this has not been directly demonstrated. The situation is similar with respect to angiotensin and neuropeptide Y; although receptors have been demonstrated in gill tissue (Tierney et al., 1997b; Bjenning et al., 1989, 1993), direct demonstration of vasoactive effects on elasmobranch gills are lacking.

Several autocrine/paracrine mechanisms (prostaglandins, adenosine, and endothelins) also modulate gill blood flow; although it is the latter two that appear to be the most potent for affecting gill vasculature in teleosts (Olson, 2002a,b). Prostaglandins produce a transient vasoconstriction and larger dose-related vasoconstriction in isolated perfused heads and single gill arches in the catshark (Piomelli et al., 1985), although to the best of our knowledge the cellular mechanisms involved in these responses remain undescribed. Adenosine causes biphasic vasoconstriction and vasodilation (depending on dose) in isolated branchial vasculature in spiny dogfish, presumably through interactions with A_1 and A_2 receptor types as in other vertebrates (Pellegrino et al., 2005; Tabrizchi and Bedi, 2001). The effects of adenosine, however, appear to be mediated by a mechanism involving NO, in that NOS inhibition abrogates both responses (Pellegrino et al., 2005). This, in turn, implies the activation of eNOS in the endothelial cells by adenosine (Fig. 1.9). But as described previously, the existence of eNOS in fish remains in question (Olson and Donald, 2009). The cellular locations (endothelium or smooth muscle cells) of the adenosine receptors, and the intracellular mechanisms by which adenosine affects fish gills, remain to be elucidated. Endothelin receptors and neuroepithelial cells containing endothelins have been identified in the gills of several elasmobranch species

(Zaccone et al., 1996; Evans and Gunderson, 1999) and we presume these compounds produce potent vasoconstrictor responses in elasmobranch gills, as it does in teleosts (Olson, 2002a).

5. THE ACTION POTENTIAL AND EXCITATION–CONTRACTION (EC) COUPLING IN ELASMOBRANCH HEARTS: THE INFLUENCES OF ENVIRONMENTAL, BIOCHEMICAL, AND MOLECULAR FACTORS

The mechanisms involved in generating the action potential and the processes involved in excitation-contraction (EC) coupling in vertebrate hearts (including fishes) are described in detail by Bers (2002), Galli and Shiels (2012), and Gamperl and Shiels (2014) and their extensive discussions will not be repeated here. Rather we will primarily concentrate on properties unique to the elasmobranch myocardium.

5.1. The Action Potential

Although elasmobranchs have received far less attention than other vertebrate groups, available data on the mechanisms underlying the action potential and EC coupling in spiny dogfish myocardial cells largely fit the accepted models for vertebrates (Fig. 1.11; Maylie et al., 1979, 1994; Maylie and Morad, 1979, 1981, 1995; Nábauer and Morad, 1992; Bers, 2002). In brief, the stable resting potential in spiny dogfish myocytes (~ -80 mV, Maylie and Morad, 1981) is within the range of those recorded in teleosts (-70 to -90 mV, Gamperl and Shiels, 2014) and is maintained through outward diffusion of K^+ (through inward rectifier channels) and the inward diffusion of Na^+ (through specific voltage-gate channels) being matched by the activity of Na^+-K^+ ATPase (Fig. 1.11B). Contraction is initiated by the action potential (Fig. 1.11A) in the pacemaker cells which spreads across the myocardium presumably through a defined (albeit as yet undescribed) specialized conduction system. We assume the pacemaker cells in elasmobranchs reside in the atria-ventricular junction and function as they do in teleosts (e.g., Haverinen and Nornanen, 2007; Icardo and Colvee, 2011; Icardo, 2012). Although to the best of our knowledge, the location and physiological characteristics of pacemaker cells in elasmobranch hearts remain undescribed.

The myocardial action potential in spiny dogfish (and by extension presumably in other elasmobranchs) is similar in shape to that seen in other vertebrates (Fig. 1.11A) and controls the contraction-relaxation cycle in

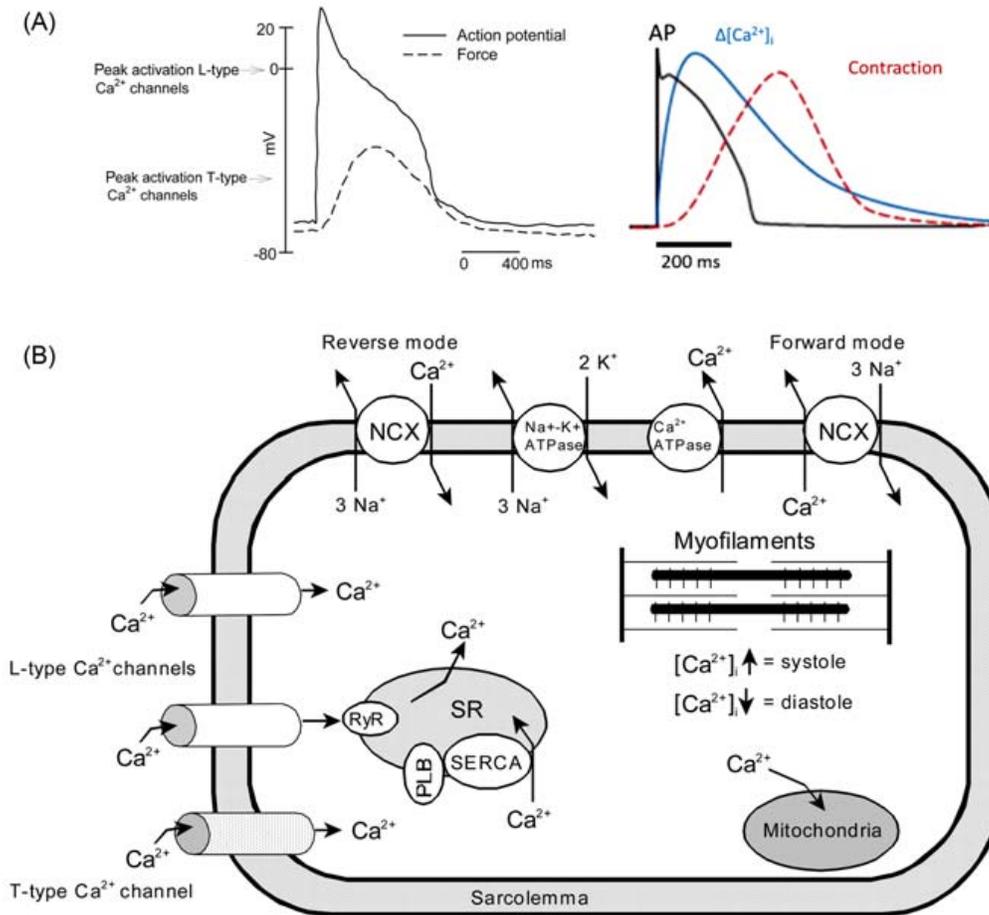


Figure 1.11. (A, left panel) The action potential (shown by membrane potential) and accompanying force of contraction of a ventricular strip isolated from spiny dogfish (at 20°C). Data are digitized from Fig. 1.1 in Maylie and Morad (1981). (A, right panel) Time course of the action potential, intracellular calcium concentration ($[Ca^{2+}]_i$), and accompanying force of a myocyte isolated from rabbit ventricle (at 37°C). Figure is from Gamperl and Shiels (2014), originally drawn by G. Galli, reproduced by permission of H. Shiels, Faculty of Medical and Human Sciences, The University of Manchester, Manchester, U.K. (B) Schematic representation of processes involved in excitation contraction coupling in elasmobranch myocytes. PLB, phospholamban; NCX, Na⁺-Ca²⁺ exchanger; RYR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum; SR, sarcoplasmic reticulum.

shark myocytes through its effect on intracellular Ca^{2+} levels (Maylie et al., 1979, 1994). The upstroke of the action potential in spiny dogfish ventricular myocytes results from the influx of Na^+ due to an increase in Na^+ conductance (resulting from the opening of the tetrodotoxin-sensitive voltage-gated Na^+ channels) accompanied by the influx of Ca^{2+} ; the latter occurring through both voltage-gated T-type and L-type Ca^{2+} channels

(Maylie and Morad, 1981, 1995). T-type Ca^{2+} channels have been shown to activate (i.e., to increase their conductance) at membrane potentials near -70 mV, with peak conductance at -34 mV; in contrast L-type Ca^{2+} channels activate near -50 mV, with peak conductance at -0 mV (Fig. 1.11A). T-type Ca^{2+} channels therefore activate essentially at the start of membrane depolarization (Fig. 1.11A) and at membrane potentials -20 mV below those resulting in an increase of conductance of the voltage-gated Na^+ channels (i.e., sooner during the upstroke of the action potential) (Maylie and Morad, 1981, 1995). It appears, therefore, the inward movements of both Na^+ and Ca^{2+} contribute to the initial rapid upstroke of the action potential. While L-type Ca^{2+} channels are well documented in teleost myocytes (Shiels, 2011; Gamperl and Shiels, 2014), the function of T-type Ca^{2+} channels remains controversial, although they have been reported to have a significant role in EC coupling in myocytes isolated from adult zebrafish (*Danio rerio*) (Nemtsas et al., 2010). As in teleosts, the inward calcium current through L-type Ca^{2+} channels is responsible for the overshoot of the action potential and the following plateau phase (Fig. 1.11A). This, in turn, activates (at a threshold of -20 mV) an outward potassium current through voltage-gated K^+ -channels, which cause the overshoot to fall of back toward zero and (at least in part) to slow repolarization during the plateau phase (Maylie and Morad, 1981; Maylie et al., 1994). The rapid fall of the action potential at the termination of the plateau phase implies the existence of a second set of voltage-gated K^+ -channels that allow the more rapid efflux of potassium and thus cause the membrane potential to fall back to its resting level. Such K^+ -channels have been described in myocytes from spiny dogfish (Maylie et al., 1994; Woo and Morad, 2001a) and appear equivalent to those described in teleost myocardial cells (Vornanen et al., 2002a).

5.2. EC Coupling

Elasmobranch cardiac muscle cells (i.e., myocytes) are morphologically similar to those of teleosts in that they are small ($\sim 2\text{--}6$ μm in diameter, or about one tenth the diameter of mammalian myocytes) and lack a T-tubule system (Maylie et al., 1979; Tota, 1989; Gamperl and Shiels, 2014). Elasmobranch myocytes have a less well developed SR than mammalian cells (Santer, 1985; Thomas et al., 1996), although some authors have described the anatomical presence (in electron micrographs) of the SR in myocytes from spiny dogfish as “frequently observed” and “considerable” (Maylie et al., 1979). In all vertebrate hearts, contraction is initiated by the rise in intracellular (i.e., cytoplasmic) free calcium ($[\text{Ca}^{2+}]_i$) which, in turn, binds to troponin C causing a conformational change that allows the

interaction of the actin-myosin filaments, which results in myofibrillar contraction (described in detail by Bers, 2002; Shiels, 2011; Gamperl and Shiels, 2014). The strength of contraction is therefore directly related to the rise in $[Ca^{2+}]_i$ (Fig. 1.11B).

In vertebrate hearts, $[Ca^{2+}]_i$ can derive from both internal (calcium-induced-calcium-release from the SR) and external (trans-sarcolemmal) sources (Fig. 1.11B). In mammals, the former predominates (supplying ~70–90% of the rise in $[Ca^{2+}]_i$), whereas in teleosts the latter predominates (supplying ~60 to 90% of the rise in $[Ca^{2+}]_i$), with the relative contribution of each being dependent on species, tissue (atria or ventricle), and temperature (Tibbits et al., 1991; Galli and Shiels, 2012; Gamperl and Shiels, 2014). While not as extensively investigated as in teleosts, the general consensus is that in elasmobranch myocytes (because of their small size and lack of a T-tubule system) trans-sarcolemmal calcium movement is sufficient, and that calcium-induced-calcium-release from the SR plays a minor role in EC coupling (Maylie et al., 1979; Thomas et al., 1996). Supportive of the conclusion that trans-sarcolemmal calcium movement predominates in elasmobranchs is the observation that contraction of ventricular myocytes isolated from spiny dogfish is blocked by nifedipine (a calcium-channel blocker primarily affecting voltage-gated L-type calcium channels). This result indicates that ($[Ca^{2+}]_i$) derives from external sources, and significant contribution of calcium released from the SR is not required (Näbauer and Morad, 1992). The two exceptions of which we are aware are: (i) the report of significant intracellular calcium storage capability in the ventricle of the little skate (Driedzic and Gesser, 1988) and (ii) enhanced Ca^{2+} uptake into the SR via the sarco/endoplasmic reticulum Ca^{2+} -ATPase mechanism (SERCA, Fig. 1.11B) in salmon shark (*Lamna ditropis*) myocardium (Weng et al., 2005). The latter instance implies significant SR involvement in EC coupling that (as discussed more fully below) is permissive of this species' subarctic-to-subtropical niche (i.e., occupancy of water from 2° to 24°C) (Weng et al., 2005).

The second route for calcium movement is the Na^+ - Ca^{2+} exchanger (NCX), which transfers three sodium ions and one calcium ion in opposite directions across the sarcolemma (Fig. 1.11B). More importantly, the NCX can operate in a bimodal fashion. When the myocyte is at rest (i.e., during ventricular diastole), the NCX is poised to operate in the “forward” or calcium-extrusion mode and therefore to move calcium out of the cell. Over the range of membrane potentials occurring during the action potential (~0–10 mV; Fig. 1.11A), however, the electrochemical driving gradient for sodium causes the NCX to operate in the “reverse” mode favoring movement of calcium into the cell (Bers, 2002). The NCX operating in reverse mode has been shown to play a major role in increasing $[Ca^{2+}]_i$

(and therefore controlling contraction) in both teleost (Vornanen, 1999; Hove-Madsen et al., 2000, 2003) and elasmobranch myocytes (Näbauer and Morad, 1992; Janowski et al., 2007). This is unlike the situation in mammals where the NCX plays a minor role in EC coupling (Morad et al., 2011).

5.3. Effects of Catecholamines and Acetylcholine

The molecular basis for the effects of adrenergic and cholinergic stimulation on vertebrate myocardium is becoming understood down to the molecular level (e.g., Hartzell, 1988; Harvey and Belevych, 2003; He et al., 2003), and this extends even to elasmobranchs (Tong et al., 2003; Janowski et al., 2007, 2009; Day et al., 2004, 2006; Cleemann et al., 2006). In mammals, adrenergic stimulation increases myocardial contraction (positive inotropic effect) and accelerates relaxation (positive lusitropic effect) through stimulation of cyclic adenosine monophosphate (cAMP), subsequent activation of protein kinases, and eventual phosphorylation of several related proteins involved in EC coupling (described in detail by Bers, 2002). In myocytes isolated from spiny dogfish, application of isoproterenol (β -adrenergic agonist) increases calcium influx through the L-type calcium channels (through protein kinase mediated phosphorylation), reduces the voltage of peak conductance from ~ 0 mV to ~ -10 mV, and prolongs the plateau phase of the action potential (Maylie and Morad, 1981, 1995). All of these effects enhance the rise in $[\text{Ca}^{2+}]_i$ and therefore explain the positive inotropic effect resulting from the stimulation of β -adrenergic receptors in elasmobranch myocardium described previously. Adrenergic stimulation is apparently without effect on T-type calcium channels, however (Maylie and Morad, 1995).

Adrenergic stimulation has also been shown to have an unusual bimodal effect on the spiny dogfish myocardium NCX through protein kinase-mediated phosphorylation. Phosphorylation enhances NCX operation in the forward mode (i.e., moving Ca^{2+} out of the cell, Fig. 1.11B), while simultaneously suppressing its operation in the reverse mode (i.e., bringing Ca^{2+} into the cell during the plateau of the action potential) (Woo and Morad, 2001b; Cleeman et al., 2006; Haviland et al., 2008; Janowski et al., 2007, 2009). The net result is a more rapid rise and decay in $[\text{Ca}^{2+}]_i$, which is the mechanistic explanation for the positive inotropic and lusitropic effects of adrenergic stimulation observed in spiny dogfish myocardium (e.g., Van Vliet et al., 1988). The bimodal effect of adrenaline on the NCX provides a mechanistic explanation for how adrenergic stimulation can have positive inotropic and lusitropic effects in elasmobranch myocardial cells that presumably lack the functional SR found in mammalian myocytes, and

where intracellular (i.e., SR) calcium release and uptake functions are enhanced by adrenergic stimulation (described in detail by [Bers, 2002](#)).

The effects of acetylcholine on elasmobranch myocyte calcium kinetics are much less studied than those of β -adrenergic receptor stimulants. Available data show, however, that acetylcholine concentrations from 10^{-6} to 10^{-4} M have no influence on inward calcium movements through T- or L-type Ca^{2+} channels, with the possible exception that acetylcholine can reduce calcium movements through the former in the presence of isoproterenol (β -adrenergic agonist) ([Maylie and Morad, 1995](#)). The negative chronotropic response to acetylcholine seen in elasmobranch hearts is presumably through some as yet undescribed influence on cardiac pacemaker cells.

Finally brief mention should be made of the effects of *in situ* generated NO modulating cardiac contractility, sensitivity to end diastolic volume (Frank–Starling mechanism), and responses to adrenergic (adrenaline) and cholinergic (acetylcholine) stimulation. Although well studied in mammalian and teleost myocardium (reviewed by [Tota et al., 2005](#)), the issue remains unstudied in elasmobranchs in spite of the evidence that these are phylogenetically ancient mechanisms. Clearly this could be a fruitful area of investigation.

5.4. Effects of Temperature and Acidosis

We end this subsection by briefly highlighting important aspects of myocardial function that are well studied in teleosts, but that remain largely un-investigated in elasmobranchs. The first is the effects of acute and seasonal temperature change on EC coupling. The reviews of [Vornanen et al. \(2002b\)](#) and [Gamperl and Shiels \(2014\)](#) describe the extensive amount of work done on the effects of temperature myocardial function and myocardial compensatory mechanisms, but all of it is on teleosts. The same can be said of myocardial force-frequency relationships. The extensive review of this topic by [Shiels et al. \(2002\)](#) contains only one reference to elasmobranchs, that of [Driedzic and Gesser \(1988\)](#). Likewise, the role of adrenergic stimulation in maintenance of cardiac function in the face of acute reductions in temperature, hypoxia, and acidosis (well described in teleosts and reviewed by [Gamperl and Shiels, 2014](#)), remains undescribed in elasmobranchs. As pointed out by [Janowski et al. \(2007\)](#), functional properties of the important NCX in shark myocytes are likely affected by phosphorylation (i.e., by catecholamines), temperature, etc., yet all these remain unstudied.

6. PRACTICAL APPLICATIONS: PHYSIOLOGY IN THE SERVICE OF ELASMOBRANCH CONSERVATION

Even though the unique features of elasmobranch cardiac physiology have applicability for improving our understanding of human heart disease (Dowd et al., 2010; Tota et al., 2011; Hickey et al., 2012), elasmobranchs are more than just biomedical models or subjects of intellectual curiosity. They are important members of aquatic ecosystems (e.g., Ferretti et al., 2008, 2010). Yet numerous elasmobranch populations are depleted due to species-specific life history characteristics (e.g., late sexual maturity and low reproductive rates) (Smith and Snow, 1998; Chen and Yuan, 2006; Snelson et al., 2008) and unsustainable rates of fisheries-associated mortality (Camhi, 2009; Garcia et al., 2008; Dulvy et al., 2008, 2014; Filmalter et al., 2013; Worm et al., 2013). Indeed, some elasmobranch species are currently considered at risk of extinction (Gallagher et al., 2012). This situation is further exacerbated by environmental challenges imposed increasing coastal hypoxia, global climate change, and ocean acidification. Following the lead of Pörtner and Knust (2007), Pörtner and Farrell (2008), Farrell et al. (2009), Seebacher and Franklin (2012), and Coristine et al. (2014), we will first briefly describe how these challenges intersect with elasmobranch cardiovascular physiology; affect their aerobic scope and eventually their roles in the community and ecosystems within which they reside; and why mechanistic and quantitative understanding of these processes are needed. We also suggest avenues for future research. Following the lead of Molina and Cooke (2012) and Cooke et al. (2013), we also describe how a mechanistic understanding the physiological effects of capture and the cardiovascular physiology of elasmobranchs can be useful for reducing elasmobranch bycatch in the world's fisheries and subsequent post-release mortality.

6.1. Global Climate Change and Ocean Acidification

We argue that understanding cardiorespiratory performance, and particularly the effects of temperature, is important in two areas: (i) understanding rates of post-release mortality and species-specific vertical mobility patterns in the large pelagic elasmobranch (e.g., blue shark, *Prionace glauca*; silky shark, *Carcharhinus falciformis*) that frequently interact with commercial fishing gear (e.g., Campana et al., 2009b; Filmalter et al., 2013; Poisson et al., 2014a) and (ii) predicting the effects of directional climate change. The former is particularly critical with respect to devising best handling practices and fishing gear configuration – deployment

strategies that effectively reduce shark bycatch and rates of post-release mortality (e.g., [Erickson and Berkley, 2008](#); [Beverly et al., 2009](#); [Poisson et al., 2014a,b](#)), which we describe in the following section. The latter is obviously required for predicting the effects of rising temperatures on the scope of activity (i.e., the difference between minimal and maximal metabolic rates and within which all life's process must occur) ([Lefrançois and Claireaux, 2003](#); [Del Raye and Weng, 2015](#)). [Farrell \(1997, 2002\)](#) has reviewed the limiting effects of higher temperature on fish maximum cardiorespiratory performance and the scope of activity that, in salmonids at least, appears largely due to the decrease in oxygen supplied to the myocardium by the venous blood. But as described in [Section 2.1](#), in elasmobranchs the venous blood may be a minor component of oxygen supply to the myocardium, with the majority being delivered by arterial blood carried in the coronary arteries. As a result, the effects of temperature on myocardial function *in vivo* may well be different in teleosts and elasmobranchs. Yet to the best of our knowledge the effects and interactions of temperature, hypoxia, and exercise on cardiorespiratory performance in elasmobranchs remain unstudied.

6.2. Surviving Interactions with Fishing Gear

Although elasmobranchs usually are not directly targeted, they do comprise a large component of bycatch in commercial fisheries worldwide ([Oliver et al., 2015](#)), particularly the large pelagic sharks captured by longline and purse seine fisheries targeting tunas ([Gilman et al., 2008](#); [Gallagher et al., 2014b](#); [Poisson et al., 2014a](#)). The fate of captured individuals is of concern because of severe shark population declines and the resulting cascading ecological effects (e.g., [Baum et al., 2003](#); [Myers et al., 2007](#); [Camhi, 2009](#); [Ferretti et al., 2010](#)). The harsh conditions to which sharks captured during purse seine operations are subjected result in high rates of both at-vessel and post-release mortality (72% and 48%, respectively) ([Poisson et al., 2014a](#)). In longline fisheries, the at-vessel mortality ranges from zero to 100% depending primarily on shark species. But it is also affected by area, gear configuration, and other factors (e.g., [Campana et al., 2009a](#); [Musyl et al., 2011](#); [Bromhead et al., 2012](#); [Gallagher et al., 2014a,b](#), [Poisson et al., 2014b](#)). Moreover, at-vessel mortality rates in longline-captured sharks differ markedly even among members of the same genera (*Carcharhinus*): sandbar shark (36%), silky shark (66%), dusky shark (*C. obscurus*, 49–81%), night shark (*C. signatus*, 81%), and blacktip shark (*C. limbatus*, 88%) ([Beerkircher et al., 2002](#); [Morgan and Burgess, 2007](#)). The same can be said about rates of post-release mortality ([Moyes et al., 2006](#); [Campana et al., 2009b](#); [Musyl et al., 2011](#); [Gallagher et al., 2014](#)).

Yet in spite of a plethora of studies using various hematological and other parameters aimed at understanding and predicting at-vessel and post-release mortality rates (e.g., [Hight et al., 2007](#); [Skomal, 2007](#); [Skomal and Bernal, 2010](#); [Marshall et al., 2012](#), and the accompanying papers in the same issue of the journal), there is yet no clear mechanistic understanding for the species-specific differences in the ability of elasmobranchs to survive the prolonged immobilization and the resultant hypoxia due to restricted water flow over the gills, which often accompany capture in ram-ventilating species (e.g., [Frick et al., 2010](#); [Gallagher et al., 2014](#)). We hypothesize the answer may well lay in differences in the ability the cardio-respiratory systems of various elasmobranch species to maintain adequate function in the face of severe acid–base and electrolyte disturbances known to accompany capture (e.g., [Brill et al., 2008](#); [Mandelman and Skomal, 2009](#); [Marshall et al., 2012](#)); more specifically the ability of various species to maintain oxygen delivery and myocardial function in the face of severe plasma acidosis. We presume elevated plasma catecholamines have an important role in maintaining myocardial contractility in elasmobranchs under these conditions as they do in teleosts (e.g., [Farrell et al., 1983](#); [Farrell, 1985](#); [Hanson et al., 2006](#); [Hight et al., 2007](#)). But to the best of our knowledge this remains uninvestigated in elasmobranchs. Likewise, longline caught sharks generally have elevated levels of plasma calcium (>3 mM) compared to unstressed sharks (<1 mM) ([Marshall et al., 2012](#)), which may help maintain contractility in the face of the accompanying plasma acidosis and elevated plasma potassium levels ([Driedzic and Gesser, 1988](#)). Although again to the best of our knowledge this too remains unstudied. Elasmobranchs also show species-specific changes in plasma potassium in response to capture. These range from no change, to an approximate doubling (from normal levels of ~ 4 mM to >10 mM post-capture; [Marshall et al., 2012](#)). But the effects of the changes in plasma potassium on elasmobranch myocardial EC coupling, and cardiac function overall, also remain unstudied.

Obviously the best way to mitigate the problems associated with shark bycatch is to minimize interactions with fishing gear altogether. In the case of pelagic longline fisheries, this can be accomplished either (i) through appropriate gear modification so as to create a mismatch between the depths occupied by the baited hooks and the sharks' vertical distribution in the water column (e.g., [Beverly et al., 2009](#)), or (ii) through the use of an effective (but selective) repellent that takes advantage of specific differences in the sensory abilities between elasmobranch and the targeted teleost species (e.g., electroreception in the former). The development of effective shark repellents has, however, met with only limited success in spite of decades of effort ([Hart and Collin, 2015](#)). Development of longline gear

targeting strategies to minimize shark bycatch obviously requires an understanding of the vertical movement patterns of the various pelagic shark species, as well as how these are modified by changes in oceanographic conditions (i.e., the depths of the thermocline and oxycline). It is now well established that the vertical movements of large pelagic fishes (e.g., tunas and billfishes) are set in large measure by the ability to maintain cardiac output in the face of reductions in temperature occurring with depth (Brill and Bushnell, 2001; Blank et al., 2004; Bernal et al., 2009; Galli et al., 2009). Moreover, although far less data are available, the same is true for pelagic elasmobranchs such as salmon and blue sharks. The former has a broader subarctic-to-tropical thermal niche (2–24°C) than do blue sharks and an enhanced expression of EC proteins associated with SR function in the myocardium (Weng et al., 2005; Musyl et al., 2011). The adaptation of an enhanced SR involvement in EC coupling and the cardiac contraction-relaxation cycle over a broad range of temperature appears strikingly similar in elasmobranch and teleost myocardium (Blank et al., 2004; Landeira-Fernandez et al., 2004, 2012; Castilho et al., 2007; Da Silva et al., 2011; Galli et al., 2011). This suggests that the selective pressures for maintaining cardiac function over a broad range of temperatures in teleosts and elasmobranchs has produced a remarkable degree of convergent evolution. Yet while this topic has been well explored in the former, it remains almost unstudied in the latter in spite of the importance of understanding the effects of temperature on cardiac function in elasmobranchs for predicting the vertical movements, vulnerability to capture by specific fishing gears, and the effects of global climate change (Del Raye and Weng, 2015). Obviously we encourage further investigations in this area.

7. SUMMARY

Although elasmobranchs occupy almost all aquatic environments and show great diversity in morphology, feeding strategies, energetics, life style, ecology, etc., the functional properties of the cardiovascular systems of only relatively few species have been investigated. This situation results, in large measure, from the limited access to live specimens. Nonetheless some general principles have emerged. In teleost and elasmobranch species, there are no particular functional characteristics distinguishing the cardiovascular systems in the two groups at routine metabolic rates. Differences do become apparent, however, when comparing cardiovascular function of high-energy-demand species (e.g., mako shark and yellowfin or skipjack tunas) at maximum metabolic rates. In this case, the lower maximum heart

rates (and therefore presumably maximum cardiac output) and the apparent inability of elasmobranchs to increase hematocrit (and therefore maximum blood oxygen carrying capacity) limit maximum metabolic rates of mako shark to about one fifth those of tunas. The limited ability to increase hematocrit (i.e., to increase maximum blood oxygen carrying capacity) in turn has significant impacts on multiple aspect of elasmobranch biology.

Although measurements are limited to just a few species, elasmobranchs appear to have a venous PO_2 below that needed for adequate rates of O_2 diffusion from the venous blood to myocardial cells (a minimum PO_2 of 1.3 kPa). As a result (and in contrast to teleosts), elasmobranch venous blood cannot be a significant source of oxygen supply to the spongy myocardium. Elasmobranchs therefore have capillary vascularization of the myocardium and thebesian vessel-like shunts connecting the arterial oxygenated coronary blood vessels to the venous lacunary system of the spongy myocardium.

Elasmobranchs have an autonomic nervous system separable into parasympathetic and sympathetic components. Activity in the vagus nerve has a major role in controlling heart rate (negative chronotropic effect), but sympathetic innervation of the heart is absent. Rather circulating catecholamines (released by chromaffin cells into the anterior venous system) are the sympathetic mechanism controlling cardiac function. Elasmobranchs appear to increase cardiac output primarily by increasing stroke volume. Stroke volume is, in turn, primarily determined by ventricular end diastolic volume; which is determined by filling time (i.e., diastolic interval) and venous return. This latter is determined primarily by venous filling pressure (also called central venous pressure), which is now known to be influenced by α -adrenergic (constrictive) and β -adrenergic (dilatory) responses of the venous system that influence venous tone and therefore venous capacitance. Changes in these parameters influence venous return, stroke volume, and cardiac output. Venous return in elasmobranchs was, at one time, considered to be influenced by significantly negative pericardial pressure resulting from ventricular blood ejection occurring from within a structurally rigid pericardial chamber. More recent observations have shown this not to be the case, however.

As in other vertebrates, blood volume and blood pressure in elasmobranchs are regulated by a complex of endocrine (which includes renin-angiotensin, kallikrein-kinin, and natriuretic peptides) and paracrine (endothelins, prostaglandins, the gasotransmitters NO and H_2S) signaling mechanisms effecting vascular smooth muscle cells and therefore blood vessel diameter, including those of the gills. The control of the elasmobranch vasculature by autonomic nervous system is, however, less developed than in

other vertebrates and control of the vasculature is considered to be primarily by circulating catecholamines. Vasoconstrictive, vasodilatory, and biphasic responses of various vascular beds are therefore due to the presence of α - and β -adrenergic receptors. Acetylcholine also has significant vasomotor effects in elasmobranchs (as it does in teleosts) causing constriction in systemic vessels and dilation of coronary arteries; although its source and overall role *in vivo* are not well understood. Nonadrenergic noncholinergic (NANC) nerves (i.e., those releasing serotonin, vasoactive intestinal polypeptide, bombesin, and neuropeptide Y) also play roles in controlling blood flow in elasmobranchs, but again these are not yet well defined.

Mechanisms involved in the action potential and EC coupling in elasmobranch hearts largely fit the accepted model for vertebrates. Myocardial contraction is initiated by the action potential which causes the influx of Ca^{2+} through voltage-gated T-type and L-type Ca^{2+} channels. Elasmobranch myocytes have a less well developed SR than mammals, and as a result the rise in intracellular (i.e., cytoplasmic) free calcium is primarily from external (trans-sarcolemmal) sources, which includes the Na^+ - Ca^{2+} exchanger (NCX). Adrenergic stimulation increases calcium influx through the L-type calcium channels and has an unusual bimodal effect on the elasmobranch myocardium NCX, it enhances its operation in the forward mode (i.e., moving Ca^{2+} out of the cell) while simultaneously suppressing its operation in the reverse mode (i.e., bringing Ca^{2+} into the cell during the plateau of the action potential). The net result is a more rapid rise and decay $[\text{Ca}^{2+}]_i$, which is the mechanistic explanation for the positive inotropic and lusitropic effects of adrenergic stimulation seen *in vivo*. In contrast to the well-described mechanisms of adrenergic stimulation on EC coupling, the effects of temperature and acidosis on elasmobranch myocardium function, along with their mitigation by adrenergic stimulation, remain largely uninvestigated.

The shortcomings in our understanding of elasmobranch cardiovascular physiology have more than academic implications. Elasmobranchs are important members of aquatic ecosystems. Unfortunately some populations are severely depleted and some species are currently at risk of extinction due to the intersection of life history characteristics (e.g., late sexual maturity and low reproductive rates), unsustainable rates of fisheries-associated mortality, and environmental degradation. We argue that to address these issues effectively will require a better understanding of the elasmobranch cardiovascular physiology, including the ability of various species to withstand the physiological consequences of the increasing temperature and expanding hypoxic zones that accompany global climate change, and the severe acidosis and the plasma ionic imbalances resulting from interactions with fishing gear.

ACKNOWLEDGMENTS

This chapter is respectively dedicated to the late Dr. Jeffery Graham (University of California, Scripps Institution of Oceanography). He was a valued mentor, colleague, and friend. We also thank Georgina Cox, Anthony Farrell, and an anonymous reviewer for providing critical comments and helpful suggestions on earlier versions of this chapter. The opinions expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies.

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CONTROL OF BREATHING IN ELASMOBRANCHS

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This review focuses on the respiratory physiology of elasmobranchs, concentrating on the generation of the respiratory rhythm, the patterns of motor output, the sources of key sensory inputs, and the functional links between respiration and the cardiovascular system that together are responsible for the exchange and transport of respiratory gases. The structure of elasmobranch gills and the mechanisms generating the flow of water over their exchange surfaces are different from teleosts and together preclude the highly effective exchange of oxygen described for teleost gills, placing limits on their maximal swimming performance. Inspiration is passive in routinely active animals but they are able to recruit feeding muscles, innervated by hypobranchial nerves into the respiratory cycle thus generating forced inspiration. The location of the respiratory rhythm generator is as yet uncertain. The trigeminal nucleus and activity in the trigeminal (Vth) nerve seem to initiate a pattern of activity that is transmitted rostral-caudally through the sequential series of motor nuclei supplying other cranial nerves that innervate respiratory structures. This

activity may be relayed to the hypobranchial nerve complex. The central origins of respiratory rhythm generation (RRG) remain unclear; consequently, this review constructs a paradigm for RRG in elasmobranchs on the basis of studies on neonatal mammals and cyclostomes that invites critical investigation.

Effective exchange of respiratory gases across the gills is affected by exposure to hypoxia or elevated levels of CO₂ or ammonia, with receptors for each of these gases located on the gills or more diffusely over the extrabranchial surfaces of the respiratory chambers. We present evidence of central, feed-forward control of the heart in elasmobranch fishes that can cause the heart to beat in synchrony with pulsatile gill ventilation, under the control of bursts of efferent activity in cardiac branches of the Xth cranial nerve, the vagus. This nerve also transmits nonbursting efferent activity, which increases during hypoxia, and may be responsible for the observed bradycardia. This separation of function relates to the location of cardiac vagal preganglionic neurons in the brainstem and their anatomical and functional separation reflects that observed in the mammalian brain. As the elasmobranchs lack sympathetic innervation of the heart, this identifies them as a model for study of the instantaneous control of heartbeat by the parasympathetic nervous system, operating via the cardiac branch of the vagus nerve and its consequent integration with respiratory activity.

1. INTRODUCTION

There is an extensive literature on control of ventilation in fish in general (Perry et al., 2009a; Reid et al., 2005; Gilmour and Perry, 2007; Collidge et al., 2007), as well as excellent coverage of the older literature on ventilation in elasmobranchs specifically (Shelton et al., 1986; Butler and Metcalfe, 1988; Taylor et al., 1999). Our focus will therefore be on recent advances in this area; on what is known and not known about control of ventilation; and on how control of ventilation in elasmobranchs differs from that in the bony fishes. As noted by others in this volume, much of the data on elasmobranchs have been obtained from a handful of species, notably species of *Scyliorhinus* and *Squalus*, and the extent to which these data are representative of the group as a whole remains to be established.¹

¹*Scyliorhinus*, is known as the dogfish in the UK (e.g. *S. canicula* is called the lesser-spotted dogfish) but is called the catshark in North America. *Squalus acanthias*, is called dogfish in North America. We will use the North American terminology throughout this chapter.

2. VENTILATION: EFFERENT MOTOR OUTPUT TO THE RESPIRATORY MUSCLES

All fishes (i.e., both Chondrichthyes and Osteichthyes) use the contraction of muscles inserted around the skull, jaws, and gill arches for gill ventilation (Fig. 2.1). The pattern of contraction of the respiratory muscles driving gill ventilation, and the pressures they produce in elasmobranch fishes (Fig. 2.2), were first described for the catshark (*Scyliorhinus canicula*) by Hughes (1960a) and Hughes and Ballintijn (1965). In elasmobranchs the contraction phase of ventilation is active, but the expansion phase is passive during normal respiration being powered by elastic forces generated in the branchial skeleton. The respiratory muscles are innervated by motor neurons arising from the medulla (pons and caudal medulla) via a series of cranial nerves (termed the “branchial nerves”). These include the trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagus (Xth) cranial nerves (Fig. 2.3). They conduct efferent motor output to respiratory muscles, as well as afferent sensory input from the respiratory system. The respiratory muscles, however, do not have exclusively respiratory functions being involved also in prey capture, chewing, and

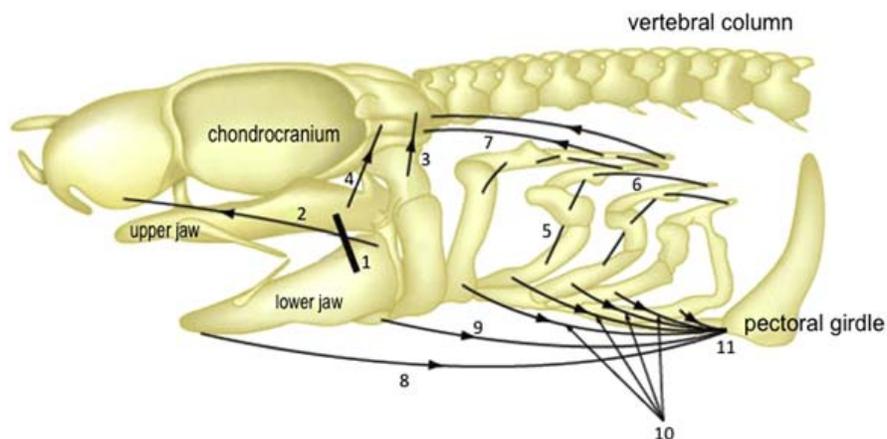


Figure 2.1. Diagrammatic illustration of the catshark skull and branchial skeleton showing the insertion of muscles. Respiratory muscles (1–7) are inserted around the jaws and gill arches and are innervated by the trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagus (Xth) cranial nerves. Feeding muscles (8–11) are inserted onto the pectoral girdle and innervated by the hypobranchial nerve complex (occipital and anterior spinal nerves). They can be used to actively expand the branchial basket during forced ventilation. Muscles are (1) adductor mandibulae, the main jaw closing muscle; (2) pre-orbitalis; (3) levator hyomandibulae; (4) levator palatoquadrate; (5) adductor branchialis; (6) interarcualis dorsalis; (7) spinalis; (8) coraco-mandibularis; (9) coraco-hyoideus; (10) coraco-branchiales; (11) arcualis communis. Relabeled from Taylor (2011a, Fig. 2.10) that was based upon Fig. 2.1 from Hughes and Ballintijn (1965).

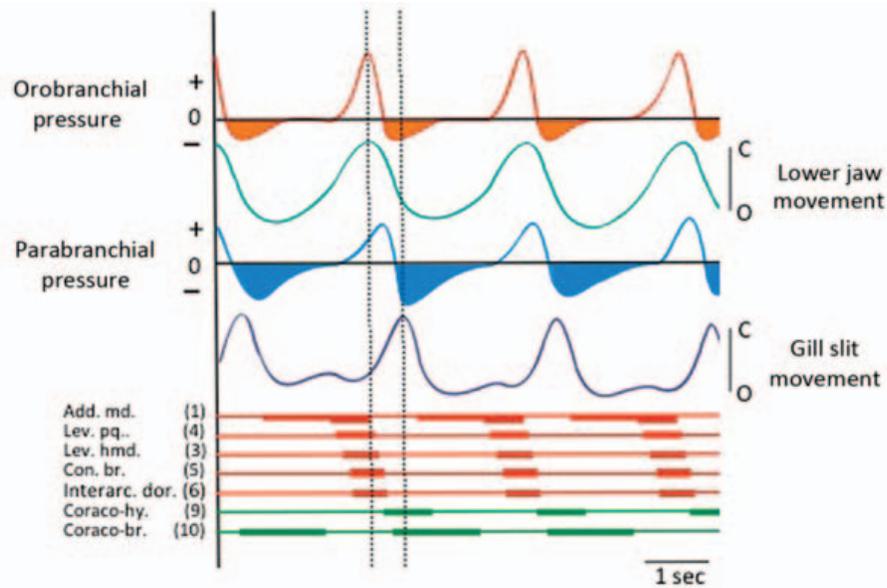


Figure 2.2. Diagram showing the pressures in the orobranchial cavity and the parabranchial cavities of the catshark, in relation to movements of the lower jaw and gill region. Below these pressures are shown the main phase of activity of muscles generating these movements. The intensity of activity in the main jaw-closing muscle, the adductor mandibulae, is indicated by the thickness of the line. The respiratory cycle is subdivided into phases in relation to the movement recordings. Redrawn from [Hughes and Ballintijn \(1965\)](#).

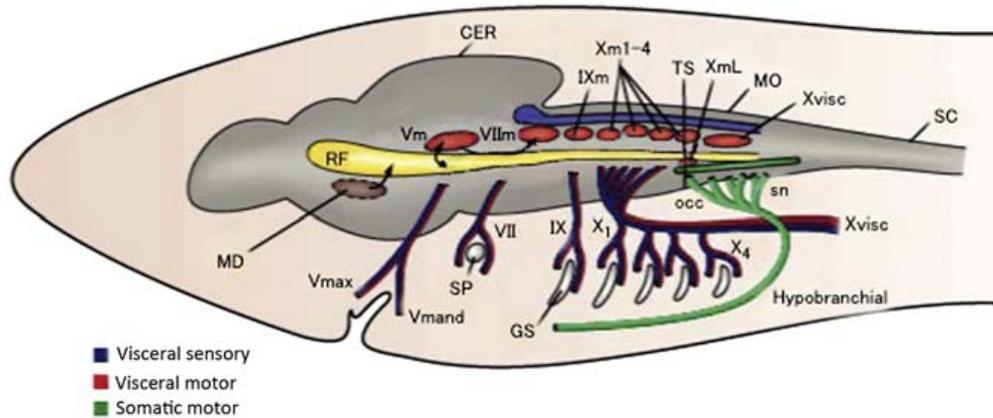


Figure 2.3. Schematic diagram of the left side of a catshark showing details of the cranial nerves innervating the respiratory system, together with the location of their motor and sensory nuclei in the brainstem. The nerves and nuclei are color coded and are listed from the most rostral to the more caudal. Red are motor nuclei: Vm, trigeminal nucleus supplying; Vmand, mandibular and Vmax, maxillary branches of Vth to jaws; VIIm, facial nucleus supplying VIIth nerve to spiracle; IXm, glossopharyngeal nucleus supplying IXth to 1st gill slit; Xm1–4, vagal (Xth) motor nuclei supplying branches of X1–4 to gill slits 2–5; Xm1, lateral nucleus of Xth supplying the heart; and Xvisc is the visceral vagus. Blue are sensory nuclei supplying trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagus (Xth) nerves. Yellow is the reticular formation (RF). Brown represents midbrain nuclei (MD), including the tegmentum. Green is occipital (occ) and anterior spinal (sn) nerves supplying the hypobranchial nerve. TS indicates position of section shown in [Fig. 2.4B](#). Arrows indicate possible interactions; details are given in the text. CER, cerebellum; GS, gill slit; MO, medulla oblongata; SC, spinal cord; SP, spiracle. Redrawn from [Taylor \(2011a, Fig. 2.1\)](#).

swallowing. The trigeminal nerve is responsible for control of the jaw-closing muscles and provides the major innervation to the mouth region of all vertebrates through the maxillary and mandibular branches to the upper and lower jaws, respectively. The facial nerve gives rise to the hyomandibular branch to branchial muscles in the hyoid arch, which includes the spiracle (an inspiratory opening in elasmobranchs), plus the levator hyoidei and the opercular muscles in teleosts. The glossopharyngeal and the vagus nerves provide efferent innervation to intrinsic respiratory muscles that control postural changes in the orientation of the gill arches during ventilation. These branchial nerves have their efferent cell bodies located dorsomedially in the brain stem, close to the fourth ventricle, in a rostrocaudally sequential series (Taylor, 1992; Figs. 2.3 and 2.4).

With the mouth-closing phase of the respiratory cycle taken as the starting point, movement recordings have revealed that there is a consistent delay in contraction from the jaws to the third gill cleft (Fig. 2.2). Simultaneous recordings of efferent activity from the central cut ends of the nerves innervating the respiratory muscles of the catshark (Barrett and Taylor, 1985a; Taylor, 1992) also reveal that the trigeminal (Vth) nerve fires in advance of the glossopharyngeal (IXth) nerve (Fig. 2.5A). This then anticipates activity in the four branches of the vagus (Xth) nerve that apparently fire together (Fig. 2.5B); although Hughes (1960a) observed slight delays in the rostro-caudal spread of the recorded pressure waves in succeeding gill clefts (Fig. 2.6). The resultant coordinated contractions of the appropriate respiratory muscles reflect the sequential topographical arrangement of the motor nuclei, including the subdivisions of the vagal motor nucleus in the hind brain of the catshark (Hughes and Ballintijn, 1965; Taylor, 1992; Taylor et al., 2009b; Figs. 2.3 and 2.4A).

The sum of these observations suggests that a sequential firing of the cranial nerves innervating respiratory muscles produces a wave of motor activity that spreads rostro-caudally through the brainstem. This, in turn, recruits the cardiac and possibly the hypobranchial nuclei (Fig. 2.4A). This may relate to the original segmental arrangement of innervation of visceral/pharyngeal clefts in protochordates before the evolution of the vertebrate brain, head, and jaws. However, this traditional view of the origin of the jaws and visceral arches and their innervation has been questioned on the basis of developmental studies of neural crest cells and marker genes (Smith and Hall, 1993). These latter results suggest a separate origin for the jaws as feeding structures independent of the visceral arches, the latter originally combining ventilation with filter-feeding. A possible evolutionary antecedent of the jaws may be the velum of filter-feeding protochordates seen in larval cyclostomes, a structure also innervated by the trigeminal (Vth) nerve (Taylor et al., 1999). The motor nucleus of the trigeminal (Vth)

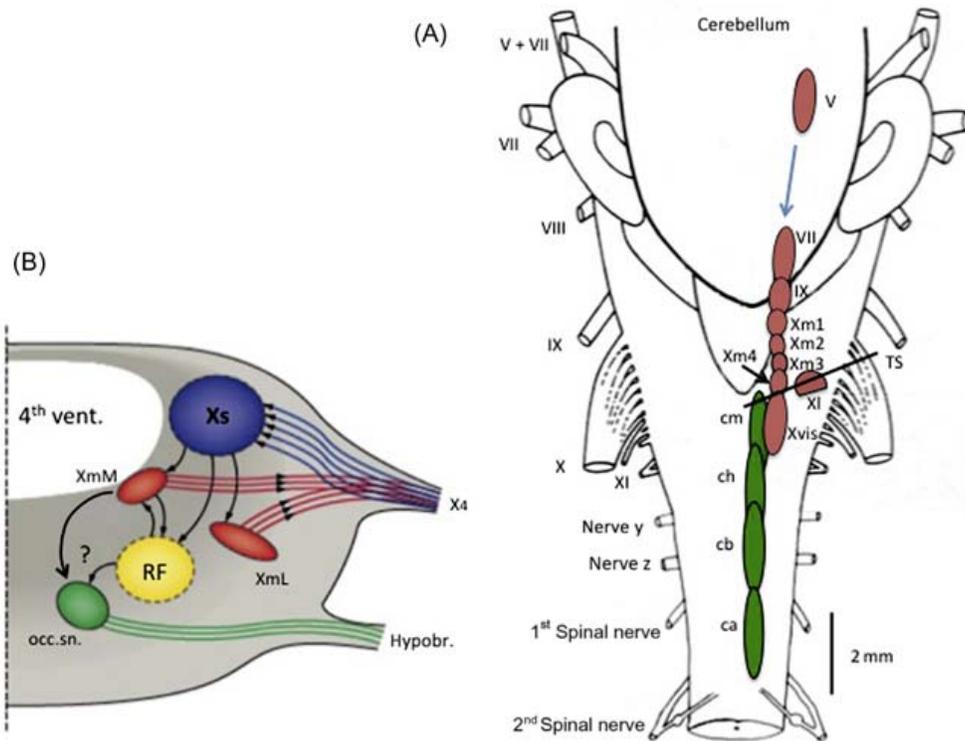


Figure 2.4. (A) Schematic dorsal view of the catshark brainstem and anterior spinal cord to show the location of motor nuclei innervating respiratory and feeding muscles. Motor nuclei to the facial (VIIth), glossopharyngeal (IXth), and vagus (Xth) nerves (red shaded areas) have an overlapping rostro-caudal, sequential distribution that may facilitate the spread of excitation from the more rostral nucleus of the trigeminal Vth nerve (the arrow suggests this connection). The target organs for these motor nuclei are listed in the caption to Fig. 2.3. The hypobranchial motor nuclei (green shaded areas) named for the muscles they innervate (see Fig. 2.1) also have an overlapping sequential distribution: cm – coraco-mandibular; ch – coraco-hyal; cb – coraco-branchiales; ca – coraco-arcual. Nerves *y* and *z* are branches of the occipital nerve, which, together with the anterior spinal nerves, contribute to the hypobranchial nerve. Redrawn from Taylor et al. (1999, Fig. 2.2) plus Taylor et al. (2006, Fig. 2.1). (B) Schematic diagram of a transverse section through the brainstem of the catshark at the point labeled on Fig. 2.3 and Fig. 2.4A (TS) to show spatial and possible functional relationships between nuclei supplying respiratory and cardiac nerves (color code and labels as in Figs. 2.3, 2.4A, and 2.12). Additional labels: 4th vent. 4th ventricle; XmL, lateral vagal preganglionic neurons; XmM, preganglionic neurons in the dorsal vagal motor nucleus; Xs, dorsal sensory nucleus of vagus; X4, 4th branchial branch of vagus. Arrows indicate possible functional connections. Details are given in text. Adapted from Taylor (2012a, Fig. 2.2).

nerve fires first in the rostro-caudal sequence in catshark and may constitute an important component of the intrinsic respiratory rhythm generator, having connections both with the reticular formation and the dorsal motor nuclei (DVN) of VII, IX, and X (Figs. 2.3, 2.4A, 2.7).

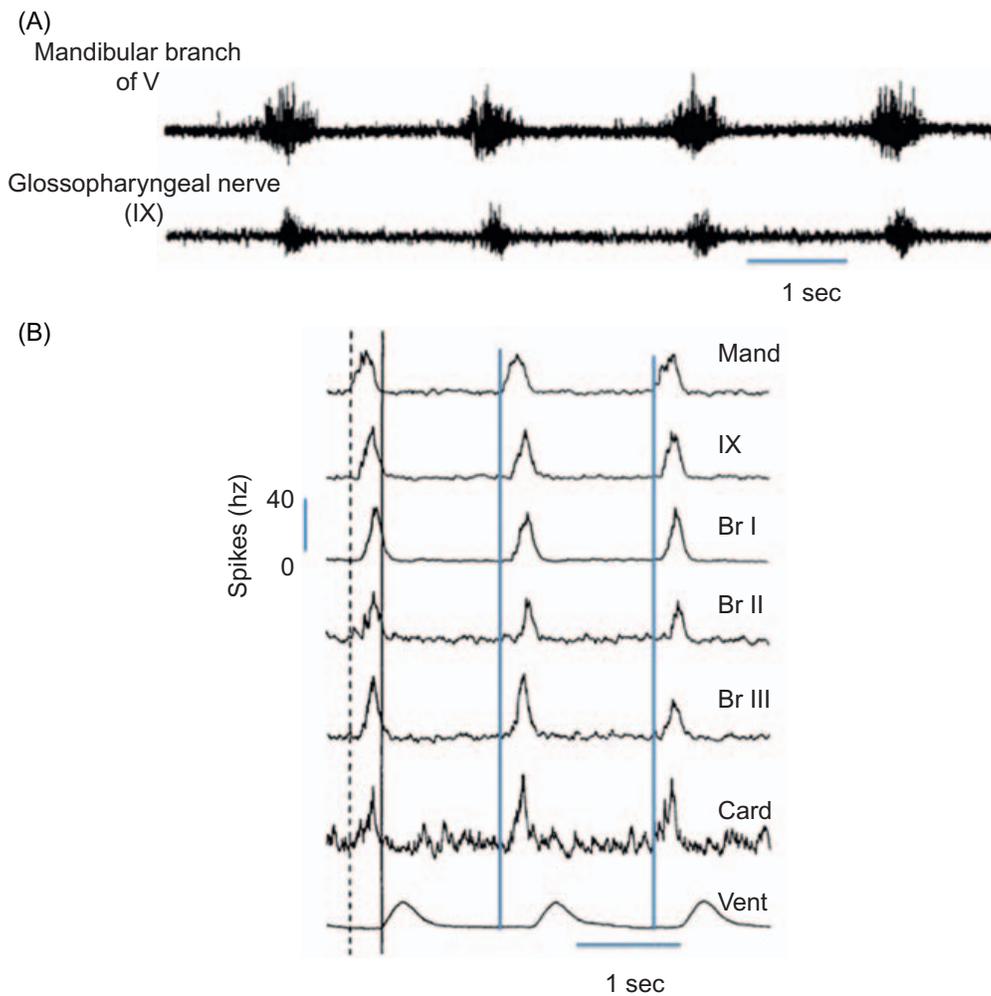


Figure 2.5. (A) Regular efferent (motor) bursting activity recorded from the mandibular Vth and the glossopharyngeal IXth cranial nerves in a spontaneously ventilating catshark. The start of a burst of activity in the mandibular Vth nerve preceded the start of a burst in the glossopharyngeal nerve by 150 ms. (B) Efferent activity recorded from the mandibular Vth (Mand); glossopharyngeal (IX); the 1st, 2nd and 3rd branchial branches of the vagus nerve (Br. I, Br. II, Br. III); and the branchial cardiac branch of the vagus nerve (Card), from the left side of the same fish together with movements of a gill septum (Vent). The recordings (cf. Fig. 2.5A) were passed through an integrator acting as a spike counter (time constant 0.1 s). The approximate spiking rate is indicated by the vertical bar. The vertical black line drawn through all the traces indicates the onset of a contraction of the first gill septum; the dashed and the blue lines indicate the start of successive bursts of activity in the mandibular nerve that preceded that in all other nerves. The bursts in the glossopharyngeal preceded those in the first, second and third branchial branches of the vagus, which occurred virtually simultaneously. The onset of respiration-related bursts in the cardiac branch often preceded those in the branchial branches and recordings from this nerve included sporadic activity between the bursts, which was absent from the respiratory nerves. From Barrett and Taylor (1985a, Figs. 2.5 and 2.6), with permission.

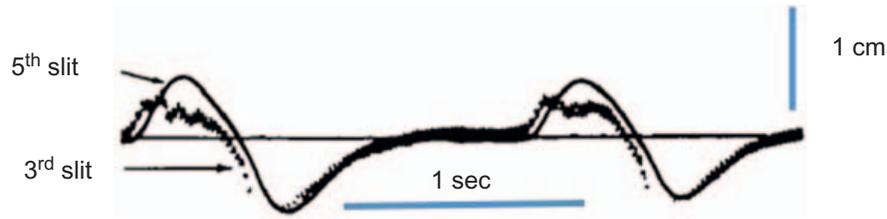


Figure 2.6. Oscilloscope records of pressure changes in the third and fifth ipsilateral parabranial cavities of the catshark, *S. canicula*. calibration = +1.0 cm water. Time = 1 s. The onset of the pressure change in the third gill slit was ahead of that in the fifth. From [Hughes \(1960a, Fig. 2.3B\)](#), with permission.

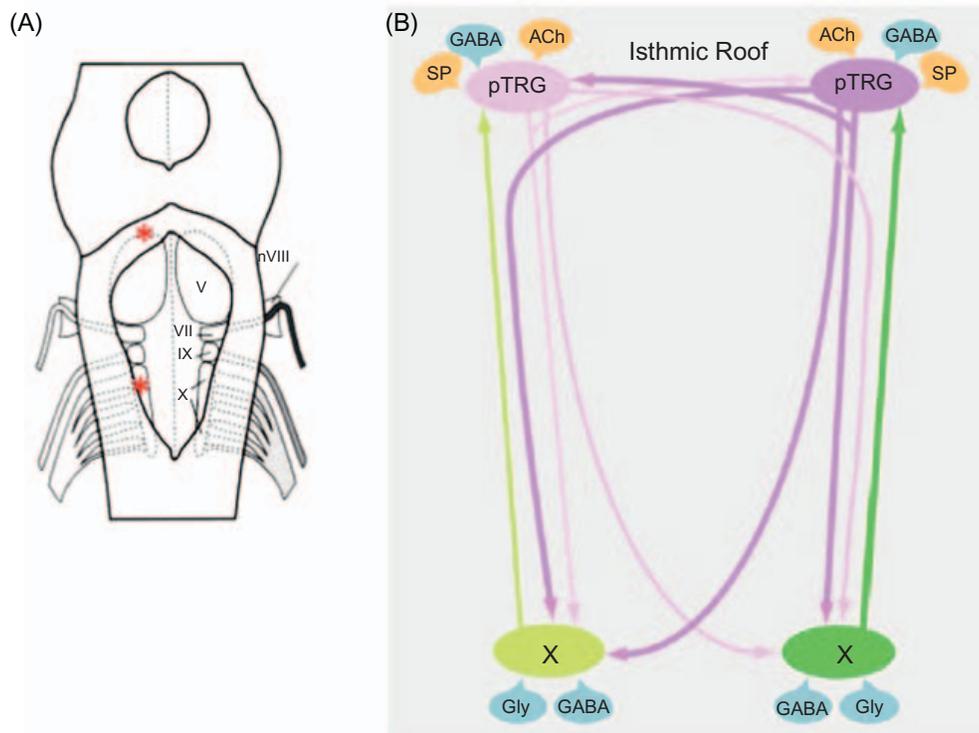


Figure 2.7. (A) Schematic illustration of the dorsal view of the lamprey brainstem. V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus. * represent areas of putative central rhythm generators (B) Schematic drawing representing findings on the connectivity within the respiratory network and relevant neurotransmitter influences. The pTRG region is shown with its projections (pink) to ipsilateral and contralateral vagal motoneuron regions and to the contralateral pTRG. Excitatory (yellow) and inhibitory (blue) influences on the pTRG region and the vagal motoneuron region are illustrated. Newly identified excitatory projections to the pTRG (green) from neurons located in the vagal area have also been reported. ACh, acetylcholine; GABA, γ -aminobutyric acid; Gly, glycine; pTRG, paratrigeminal respiratory group region; SP, substance P; X, vagal motoneuron region. From [Cinelli et al. \(2014\)](#), with permission.

3. CENTRAL RESPIRATORY RHYTHM GENERATION: THE SOURCE OF THE MOTOR OUTPUT

It is well accepted that the respiratory rhythm in vertebrates arises from populations of cells within the brainstem (Von Euler, 1986; Richter et al., 1992; Feldman et al., 2013). Over the last three decades significant advances have been made in our understanding of the location of these rhythmogenic sites, as well as of the mechanisms that underlie the generation of the rhythm particularly in neonatal rodents, amphibians, and lampreys (Feldman et al., 2013; Hedrick, 2005; Mutolo et al., 2007, 2010, 2011; Cinelli et al., 2013). Unfortunately there has been almost no research of this type on any of the Chondrichthyes or Osteichthyes. A parsimonious (but speculative) story can, however, be pieced together concerning the sites and activation sequence of rhythmogenic cells based on the nature of the motor output described in the previous section, the older data that exist for elasmobranchs and teleost fish, and the new data from other phylogenetic groups. We contend that this story can serve as the null hypothesis for future research.

Rhythm generating circuits are very common in the developing hindbrain of all vertebrates. In early neurogenesis they can be found in every pair of rhombomeres in association with the nuclei of all cranial nerves with motor outflow (Lumsden, 1990; Wingate and Lumsden, 1996). Thus, rhythm generators can be found in association with the branchiomotor components of the trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) nerves supplying the motor outflow for the first, second, and third ancestral branchial arches, respectively. The branchiomotor nucleus of the trigeminal (Vth) nerve lies in rhombomeres 2 and 3, with its cranial nerve arising from rhombomere 2. The branchiomotor and visceromotor nuclei of the facial nerve (VIIth) lie within rhombomeres 4 and 5, with their nerves exiting in rhombomere 4; while the branchiomotor and visceromotor nuclei of the glossopharyngeal (IXth) and vagal (Xth) nerves lie in rhombomeres 6 and 7 (Lumsden and Keynes, 1989). Research performed on chick embryos has demonstrated the onset of their rhythmic activities before the end of the period of segmentation (Fortin et al., 1994). Each rhythm generator could generate its own independent rhythm, with the rostral central rhythm generator (CRG) generating a faster rhythm than the more caudal CRGs. *In vivo*, they all entrain and fire together with co-activation of motor patterns resulting from intersegmental and cross-median connections between these rhythm generators (Fortin et al., 1994).

Significant to this discussion is that the respiratory pump in adult lampreys (members of the order Petromyzontiformes) is a buccal force pump driven by the very same nerves as in elasmobranchs. In lampreys, the

respiratory motoneurons are contained in the trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) nerves innervating the velum plus the muscles of the buccal pump and gills (Rovainen, 1974, 1977, 1979; Guimond et al., 2003). The ammocoete larvae of lampreys use a velar pump for unidirectional ventilation and suspension feeding. Ventilation is driven largely by the velum alone, which is innervated by the trigeminal nerve. In adult lampreys, ventilation is produced by a buccal pump moving water in and out of the separate branchial gill sacs and thus over the gills (Rovainen, 1996). This bidirectional pumping mechanism enables the adult lamprey to adhere to its host by an oral sucker and to breathe while feeding. While this is distinct from the pumping mechanism seen in elasmobranchs, which is unidirectional with water flowing in through the mouth and spiracles and out over the gills, in both groups only expiration (buccal compression) is active under resting conditions. Inspiration is completely passive due to elastic recoil (Roberts, 1950; Hughes and Ballintijn, 1965).

Selective lesion and stimulation experiments suggest that lampreys possess at least two distinct pairs of respiratory rhythm generators, one pair in the lateral trigeminal region, and one near the region of the medulla that contains the motor nuclei of the facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) nerves (Fig. 2.7A). Neurons in the lateral trigeminal region (known as the para-trigeminal respiratory group, pTRG) compose the predominant pair of breathing rhythm generators (Rovainen, 1974, 1979, 1983; Russell, 1986; Thompson, 1985; Mutolo et al., 2007, 2010, 2011; Cinelli et al., 2013). The lampreys' trigeminal pattern generators may be a holdover from the ammocoete stage, in which they drive nearby velar motoneurons as the primary pump for ventilation (Rovainen, 1996). The role of neurons in the region of the medulla that contains the motor nuclei of the facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) nerves remains uncertain. Recent data from studies using *in situ* and isolated brainstem spinal cord preparations of adult lampreys suggest that neurons at this site can produce a slow rhythm. But in these *in vitro* preparations, they receive descending drive from the pTRG and provide it with reciprocal inhibition that modulates the activity of rhythm-generating neurons in the pTRG. They do this without having any direct role in burst formation and termination mechanisms (Martell et al., 2007; Cinelli et al., 2014; Fig. 2.7B). By contrast, Kawasaki (1979, 1984) conducted similar lesion studies in semi-intact animals and found that periodic breathing movements could resume following transection to remove the pTRG. He concluded that the caudal region was indispensable for rhythm generation and suggested that it was also possible that the caudal CRG could be subdivided into separate segmental components serving the facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) nerves as a series of chain oscillators.

In elasmobranchs and bony fishes, the neurons producing respiratory rhythms are also located within the medulla, but their precise location has yet to be determined (Shelton, 1970). Removal of the fore- and midbrain (in some cases, back to the border between the mesencephalon and the midbrain) and spinal cord and posterior regions of the medulla (up to the level of obex) does not stop breathing in elasmobranchs (Satchell, 1959) or teleosts (Shelton, 1959). The discharge patterns recorded from neurons within the medulla (with respiratory-related activity) range widely, and include both neurons active during some portion of either phase of the ventilatory cycle, as well as those with phase-spanning activity. A host of studies suggest there is a longitudinal strip of nervous tissue containing respiration-related neurons extending on each side of the midline over the whole medulla oblongata (Ballintijn, 1982, 1987; Shelton et al., 1986). This strip comprises the motor nuclei of trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) cranial nerves; the descending trigeminal nucleus; the intermediate facial nucleus; and the reticular formation alongside these nuclei (Figs. 2.3 and 2.4). These data suggest that the source of rhythmic discharge comes from higher order interneurons situated close to known motoneurons. Although these neurons were reported not to be clustered according to the phase relationship between their activity and ventilation in a teleost fish (Ballintijn and Alink, 1977), measurements of sequential activity recorded from respiratory muscles and nerves of catshark (*S. canicula*) are correlated with the distribution of their motor neurons as revealed by neuranatomical studies (Barrett and Taylor, 1985a; Taylor, 1992; Figs. 2.2, 2.3, 2.4A and B, 2.5, and 2.6).

Importantly, localized destruction of cells at any rhythmically active site does not abolish the respiratory rhythm (Hyde, 1904; Shelton, 1961). This has been construed to indicate that there is either a diffuse organization of rhythm generating neurons (Waldron, 1972), or that there may be a series of coupled local oscillators (Hyde, 1904; Shelton, 1961; Ballintijn, 1982, 1987; Shelton et al., 1986). Hyde (1904), working with the skate (Genus and species not given), reported that the respiratory mechanism could be divided into two or three divisions; that each was capable of initiating coordinated rhythmical respiratory movements in the respiratory muscles to which it was segmentally related; and that each could temporarily have its own independent rhythm.

One parsimonious explanation summarizing this evidence is that both lampreys and elasmobranchs retain a series of coupled oscillators arising from the rhombomeres associated with (i.e., at least close to) the motor nuclei of the trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) cranial nerves (pTRG, pFRG, pVRG). Each rhythm generator is capable of operating independently. *In vivo*, however, they all entrain and

fire sequentially, with the co-activation of motor patterns resulting from intersegmental and cross-median connections. In both lampreys and elasmobranchs, the pTRG predominates thus giving rise to the sequential firing of the cranial nerves innervating respiratory muscles. This, in turn, produces a wave of motor activity that spreads rostro-caudally through the brainstem. We note, however, that this is an hypothesis that remains to be tested.

4. THE RESPIRATORY PATTERN: THE CONDITIONAL NATURE OF THE OUTPUT

4.1. Continuous Resting Ventilation

The gills of fishes are borne as double rows of horizontally stacked filaments on a series of pharyngeal arches supported by skeletal bone or cartilage (Fig. 2.8) (see Wilga and Ferry, 2015, for a detailed description of gills). The filaments bear on their dorsal and ventral surfaces a series of vertically stacked lamellae that effect respiratory gas exchange. In teleost fish the double rows of filaments are entirely separate, allowing water to flow between the lamellae directly from the orobranchial cavity into the opercular cavity along the whole of their length (Hughes and Shelton, 1962; Fig. 2.8C and D). Recent measurement and modeling of gill structure has revealed that interlamellar distances are similar across a wide range of teleost species despite large variations in activity levels and body mass. This implies that this spacing is optimal for the exchange of oxygen by physical diffusion from water to blood over the gills of fish (Park et al., 2014). It is well established that a countercurrent exchange mechanism functions over the gill lamellae of teleost fishes, with blood streaming through the lamellae in the opposite direction to the flow of water. This optimizes the effectiveness of gas exchange to the extent that arterialized blood can have a similar oxygen partial pressure to inspired water (Hughes and Shelton, 1962; Piiper and Scheid, 1977). In elasmobranch fishes the pairs of filaments on each gill arch are attached to a central interbranchial septum so that water passing over the filaments is deflected by the septum after it has passed between the lamellae (Fig. 2.8A and B; Cooke, 1980; Taylor, 1992; Wegner et al., 2012). The water stream then enters five separate parabronchial cavities, each open to the surrounding water via external gill slits. Each gill slit is guarded by a gill flap formed by an extension of the interbranchial septum that acts as a passive valve preventing backflow of water during expansion of the orobranchial cavity (Fig. 2.9). Despite the apparent obstruction formed by the interbranchial septum in elasmobranchs, there is evidence that functional countercurrent

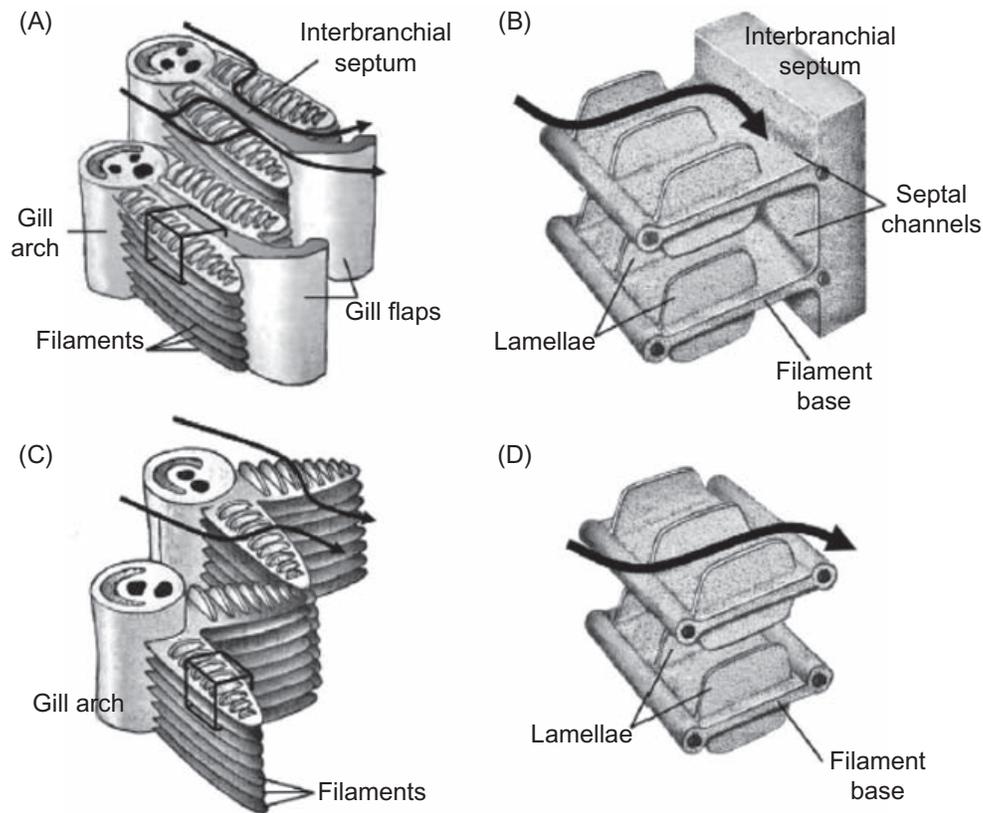


Figure 2.8. Simplified drawings of the basic structural features of the elasmobranch (A and B) and teleost (C and D) gill. B and D are enlarged views of the boxes in A and C, respectively. The path of water flow through the gills is indicated by arrows. From Wegner (2014, Fig. 2.1), with permission.

gas exchange operates over the gills due to the formation of “septal canals” that collect water as it leaves the gill lamellae before it enters the parabranial cavity (Grigg, 1970a). A detailed study of the effectiveness of oxygen transfer in the catshark (*S. canicula*), however, revealed that arterialized blood in normoxic fish had a mean oxygen partial pressure (P_{O_2}) of 14 kPa compared with the mean P_{O_2} of mixed expired water of 16 kPa and an inspired P_{O_2} of 21 kPa. Although this disparity could be related to methodological problems (Short et al., 1979), it has been established that the resistance to flow presented by the interbranchial septum may be countered by wider spacing of the gill lamellae in elasmobranchs that may reduce their effectiveness in respiratory gas exchange (Wegner et al., 2012; Wegner, 2015). This limits the aerobic scope of fast-swimming predatory fish such as the mako shark, *Isurus oxyrinchus* compared to other high energy demand pelagic species such as the tunas (see Chapter 1). We revisit limits to performance in the section on ram ventilation.

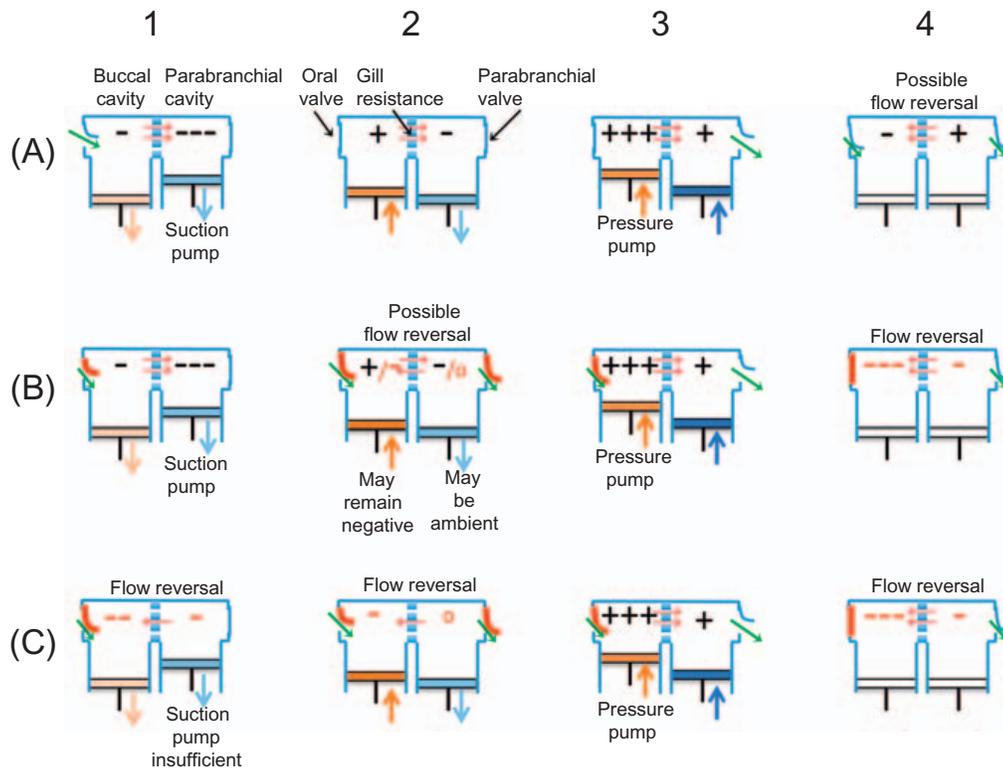


Figure 2.9. Hughes' (1965) classic model of fish respiration (A), with changes in the model to fit Hughes' (1965) data and swellshark data containing two reversals (B), and for times when the suction pump is insufficient and pressure reversals persist throughout the period of negative buccal and parabranchial pressure (C). Changes to both pressures (relative to ambient) and kinematics from the classic model are indicated in bold red markings. For example, when the oral valve is in a position different from that indicated by the classic model (A), it is shown by a thicker red line (see B, step 1). The possible flow reversal identified in A was described initially by Hughes (1965). The word "possible" is placed in quotation marks because swellshark data indicate that it is virtually always present. Redrawn from [Ferry-Graham \(1999, Fig. 2.7\)](#).

Water contains approximately 1/20th to 1/30th (depending on temperature and salinity) the amount of oxygen as an equivalent volume of air and is considerably more dense and viscous. Consequently at equivalent metabolic rates fish must generate respiratory volumes many times those of air breathing vertebrates, which requires significantly more muscular effort to meet tissue oxygen demand. To do so they normally exhibit continuous rhythmical breathing.

Water flow across the gills is produced by two cyclical pumps: one that forces water across the gills and another that draws it across ([Fig. 2.9](#)). One can therefore be active whilst the other is refilling ([Hughes, 1960b; Hughes and Shelton, 1962](#)). Water enters the open mouth drawn in by expansion of the orobranchial cavity. It is then forced out over the gills by contraction of

the pharyngeal musculature following mouth closure. During the mouth opening phase, the opercular cavity expands, drawing additional water across the gills (Hughes and Shelton, 1962). Both phases of breathing involve the active contraction of respiratory muscles and neither phase appears to be dominant (Ballintijn, 1982, 1987; Shelton et al., 1986). In many species the coordinated actions of the pre- and post-branchial pumps creates a pulsatile, albeit virtually continuous flow of water over the gills during both the mouth opening (volume expansion) and mouth closing (volume compression) phases of the ventilatory cycle (Hughes and Shelton, 1962). Pressure recordings showed only brief periods of reversal of the pressure gradient generating flow across the gills, which occurs during the initial phase of expansion of the opercular cavity (phase 4 as described by Hughes and Shelton, 1962).

Elasmobranchs lack an operculum and instead have separate septal pumps operating on the individual parabranial cavities. This confers specific properties to the patterns of flow over their gills (Wilga and Ferry, 2015). During periods of settled, routine rates of ventilation, jaw opening is passive (Hughes and Ballintijn, 1965) and only buccal compression forces water out across the gills. A suction pump is generated by elastic recoil of the branchial skeleton, although small muscles that extend the elements of the gill arch may also be partially responsible for expansion of the parabranial cavities (Hughes and Ballintijn, 1965). During this passive, relaxation phase, the gill flaps are closed so that expansion of the parabranial cavities draws water across the gill sieve from the orobranchial chamber and an almost continuous flow of water is maintained across the gills. However, this model has been challenged based on observations of extended periods of pressure reversal during the respiratory cycle in swell sharks, *Cephaloscyllium ventriosum* (Ferry-Graham, 1999). Impedance data suggest that pressures within the buccal and parabranial cavities are not generated by the cyclic opening and closing of the jaws and gills in the manner previously suggested by Hughes (1960a). Rather two alternative models of pressure generation during respiration have been described by Ferry-Graham (1999) (Fig. 2.9). In one model, the suction pump is insufficient for generating a positive pressure differential across the gills so that a pressure reversal persists throughout this phase of respiration. In the alternative model, based on study of the swell shark, a double-reversal occurs whereby pressures are reversed following both of the pump stages (the suction pump and the pressure pump) rather than after the pressure-pump stage only. However, kinematic analysis of high-speed video footage and dye studies suggested that during gill ventilation (in the absence of feeding) distinct flow reversals do not result from the recorded pressure reversals. There is also evidence that in elasmobranchs, the pressure and

suction pumps do not always work in perfect phase, leading to periods of higher pressure in the parabranchial than in the oropharyngeal cavities (Summers and Ferry-Graham, 2001). Because flow reversals were also not observed at this time, the data suggest that the gill flaps remain closed and thus water is probably pooling around the gill filaments during these periods (Ferry-Graham, 1999). In the hedgehog skate (*Leucoraja erinacea*), however, pressure reversals led to flow reversals with water flowing from the parabranchial chambers back across the gills into the orobranchial chamber (Summers and Ferry-Graham, 2001).

The pattern of water flow over the gills is further complicated by the fact that most elasmobranchs have additional inspiratory openings in the form of a pair of spiracles equipped with muscular valves. These are remnants of the gill slits between the mandibular and hyoid arches that became reduced with the development of jaws. Benthic sharks have large spiracles, while they are small or absent in pelagic species. In skates and rays that have a flattened body shape and feed from the substratum, often staying partially buried in it for long periods, the spiracles are relatively large and form the primary route for inspiratory water (Grigg, 1970b; Summers and Ferry-Graham, 2001). Studies on catshark revealed that water entering the mouth leaves through the more posterior gill slits, whereas water entering the spiracles chiefly escapes through the anterior-most two or three gill slits (Hughes and Shelton, 1962). These two points of entry do not always show clear integration. Water sometimes enters simultaneously through the mouth and spiracles, and at other times water enters either inspiratory opening alone with the mouth occasionally held open throughout the respiratory cycle (Summers and Ferry-Graham, 2001).

When its mouth is obstructed, either by contact with the substrate or during prey ingestion, the Port Jackson shark (*Heterodontus portjacksonii*) draws water into the branchial chamber through the first pair of gill slits, as well as the spiracles, the latter appearing to be too small to allow adequate flow alone. In hypoxia the first gill flaps flare open and, although they continue to beat weakly in synchrony with the others, water is drawn into the orobranchial cavity through them and is ejected from the remaining gill slits on the same side of the fish (Grigg, 1970b). Thus, the first gill slit can operate independently of the other four gill slits as a route for inspiration of water either when the mouth is obstructed during prey ingestion or during hypoxic exposure. This first gill arch is innervated by the glossopharyngeal nerve (IXth) and this may be the basis for its ability to function independently of the succeeding arches that are innervated by the vagus (Xth) nerve (Taylor, 1985). Clearly, water flow over the gills in a range of species needs to be examined to determine the extent to which any model of water flow over the gills in elasmobranchs is generally applicable.

4.2. Forced Ventilation – Recruitment of Hypaxial Muscles

Both elasmobranchs and teleosts can recruit an additional group of muscles into the respiratory cycle to provide active jaw occlusion. These are derived phylogenetically from the forward migration of four anterior myotomes (the hypaxial muscles), which come to form a complex ventral sheet of muscle that is inserted between the pectoral girdle, the lower jaw, and the ventral processes of the hyoid and branchial skeleton. These muscles are associated primarily with suction feeding and ingestion in water-breathing fishes but they can be recruited into the respiratory cycle during periods of vigorous, forced ventilation such as may occur following exercise or deep hypoxia (Hughes and Ballintijn, 1965; Ballintijn and Juch, 1984) and are used to gulp air at the water surface by many air-breathing fishes (Taylor, 2011a). Activity in these muscles was shown to be integrated into the respiratory cycle (Hughes and Ballintijn, 1965; Fig. 2.2). They are innervated by the hypobranchial nerve, which contains elements of the occipital nerves and the anterior spinal nerves (Figs. 2.3 and 2.4A and B). The hypobranchial nerve in fish is the morphological equivalent of the hypoglossal (XIIth) cranial nerve that innervates the muscles of the tongue in reptiles, birds, and mammals. These muscles are utilized in suckling by infant mammals, an activity likely to require its own central oscillator that is thought to reside in the reticular formation (Taylor et al., 1999). Similarly, in the catshark and ray (*Raia clavata*) rhythmic opening and closing of the mouth occurs during ingestion of food, which implies the central generation of a feeding rhythm. The neural mechanisms operative in the control of masticatory rhythms in fish remain unexplored, although it has been argued that the respiratory and feeding rhythms in fish, just as in other vertebrates, are generated by separate groups of interneurons (Ballintijn and Juch, 1984). It is now well established that in mammals the masticatory rhythm is generated in the hindbrain in the reticular formation, and the same has been suggested for birds (Taylor et al., 1999).

The cell bodies of neurons supplying the hypobranchial nerve are located in the ventro-lateral spino-occipital motor nucleus (Taylor et al., 2006; Figs. 2.3 and 2.4A,B). They form a continuous column of cells extending from obex in the medulla to the origin of the roots of the second spinal motor nerve (Fig. 2.4A). There is an overlapping sequential distribution of the cell bodies supplying efferent innervation to the series of ventral muscles inserted from the pectoral girdle that extend to a series of oro-pharyngeal structures (Fig. 2.1). These comprise the coracomandibular muscles that insert on the lower jaw (extending for 3 mm caudally from obex); the coracohyal muscles that insert on the basihyal cartilage (extending 4 mm from 1 mm caudal of obex); the coracobranchial muscles that insert on the

skeletal elements of the branchial arches (extending for 2.5 mm from 4.7 mm caudal of obex); and the coracoarcual muscle that inserts on Meckel's cartilage. This is a cartilaginous ventral component of the lower jaw that is innervated by hypobranchial neurons in the most caudal portion of the nucleus.

Simultaneous recordings of efferent activity in the central cut end of a branchial branch of the vagus (Xth) nerve and of a branch of the hypobranchial nerve in a decerebrate, paralyzed catshark, show that the hypobranchial nerve is relatively inactive during normal fictive ventilation (Taylor et al., 2006). Occasional spiking activity in the hypobranchial nerve always falls within a respiratory burst in the branchial nerve. Physical stimulation of the jaws or gill mechanoreceptors, or cessation of forced irrigation to stimulate chemoreceptors, induces high levels of activity in the hypobranchial nerve, separated into discrete bursts that have fixed phase relationships with respiration-related activity in the branchial branch (Taylor et al., 2006). Injection of noradrenaline to stimulate breathing (Taylor and Wilson, 1989; Randall and Taylor, 1991) also elicits activity in a previously inactive hypobranchial nerve that again appears to be respiration-related. This rapidly recovers back to low levels of activity (Taylor et al., 2006).

Recruitment of the hypaxial muscles into the respiratory cycle would result in active jaw abduction, with a potential increase in ventilation rate and volume. Central connections are possible between vagal respiratory neurons in the DVN and occipital neurons as they have an overlapping distribution in the brain stem, caudal of the obex (Taylor et al., 1999, 2006 and see Figs. 2.3 and 2.4). They thus form a possible pathway for central recruitment. This could, in turn, lead to a rostrocaudal spread of excitation, from the occipital to the anterior spinal motor nuclei, that together innervate the hypaxial muscles, similar to that observed in the series of cranial nerves supplying the respiratory muscles (Barrett and Taylor, 1985a; Taylor, 1992). This rostrocaudal spread of excitation may also be relayed to reticulomotor neurons in the catshark, which results in swimming movements of the axial musculature becoming coordinated with gill ventilation (Satchell, 1968). Future work should seek to dissect out and record from separate branches of the hypobranchial nerve that serve discrete muscles in the walls of the orobranchial cavity, to identify their patterns of firing in relation to the rostro-caudal spread of excitation from the branchial innervation, and to elucidate their roles in ventilation (Figs. 2.3 and 2.4).

4.3. Sporadic (Episodic) Ventilation

Fish often show markedly reduced ventilation rates when inactive in normoxic or hyperoxic waters. They may also interrupt their normal regular rhythm of gill ventilation and exhibit episodic breathing (Fig. 2.10). These

(A) Frequency cycling in intact animals under hyperoxic conditions.



(B) Episodic breathing under normoxic conditions following central denervation



Figure 2.10. Breathing traces from a tambaqui (*Colossoma macropomum*) that illustrate the changes in breathing pattern that arise under (A) hyperoxic conditions and (B) following central denervation of cranial nerves IX and X. Both conditions reduce or eliminate input from O_2 sensitive receptors in the gill reducing the respiratory drive. Similar responses have been reported in the Port Jackson shark. Adapted from Reid et al. (2003).

patterns, often similar to Cheyne–Stokes breathing (i.e., a waxing and waning of both breathing frequency and amplitude), have been well described in carp (*Cyprinus carpio*) (Jüch and Ballintijn, 1983; Jüch and Luiten, 1981; Lomholt and Johansen, 1979); tambaqui (*Colossoma macropomum*) (Reid et al., 2003); and the Port Jackson shark (*H. portjacksonii*) (Capra, 1976). Roberts and Ballintijn (1988) also report that breathing rhythm in a stereotaxically held decerebrate catfish shifts frequency to faster or slower rates, and that breathing amplitude often changes such that the periods of fast breathing are associated with larger amplitude breaths. Results obtained from tambaqui suggest that reduced afferent input from peripheral chemoreceptors promotes episodic breathing, but that chemoreceptors specific for producing changes in breathing pattern (versus total level of ventilation) may also exist (Reid et al., 2003). Carp possess a group of neurons with phase switching properties, situated in the midbrain, that also appear to play a key role in the control of episodic breathing. Stimulation of this area of the brainstem during a ventilatory pause brings forward the onset of the next breathing bout (Jüch and Ballintijn, 1983). Studies to determine how widespread episodic breathing is in elasmobranchs under resting conditions, especially in benthic species, would be useful, as would studies into the mechanisms underlying the production of this pattern.

4.4. Ram Ventilation

Many pelagic and mid-water fishes (e.g., remora, *Remora brachyptera*; sockeye salmon, *Oncorhynchus nerka*; and skipjack tuna, *Katsuwonus*

pelamis) can suspend active gill ventilation when moving at velocities about 1.5 km h^{-1} or higher and resort to ram ventilation of the gills (Muir and Buckley, 1967; Smith et al., 1967; Brown and Muir, 1970; Roberts, 1975). With the exception of skipjack tuna, they retain the ability to ventilate using respiratory muscles when at rest in the water column, or when maneuvering at slow speed. Most elasmobranchs are facultative ram ventilators and some are obligate ram ventilators having to swim continuously. This is particularly true of the lamnid sharks, a group that demonstrate high evolutionary convergence with tunas (a group of obligate ram ventilating teleost fish) in a variety of features related to swimming performance (Bernal et al., 2001, 2009; Shadwick, 2005; Wegner et al., 2012). The adaptive significance of this switch is likely to be the relatively high metabolic cost of oxygen uptake from water due to the low oxygen content per unit volume of water, described above. Switching to reliance on the swimming muscles for ventilation may reduce workload. There is added branchial resistance imposed by ram ventilation that increases swimming drag. For instance, ram ventilation has been shown to increase swimming drag by 9% in skipjack tuna. The drag, however, is less than the added drag that would be imposed by repeated changes in mouth gape and opercular profile during rhythmic ventilation. Consequently, the switch to ram ventilation reduced metabolic rate by 3–7% in the shark sucker (*Echeneis* spp.), and increased swimming speed by 50% with no additional metabolic cost in the striped bass (*Morone saxatilis*) and bluefish (*Pomatomus saltatrix*) (Freadman, 1981). Consequently, fish that can cruise fast enough to overcome gill flow resistance can maintain an adequate ventilatory volume to ensure complete blood oxygenation without utilizing the respiratory muscles.

The design of the elasmobranch gill creates a paradox in this regard. In all ram-ventilating species, the gills must be sufficiently rigid to resist collapse and continue to exchange gas efficiently in the forceful stream of water resulting from fast continuous swimming. In elasmobranchs, the interbranchial septum that extends between the hemibranchs of each gill arch and forms the gill flaps has been suggested to reinforce the gill and serve this purpose (Benz, 1984). The same structure, however, adds considerable resistance to branchial flow. A recent study on the shortfin mako shark, *I. oxyrinchus* (Wegner et al., 2012), demonstrated that this increase in resistance was compensated by an increase in lamellar spacing that reduced resistance to flow through the interlamellar channels. This solution to reducing flow resistance, however, reduces gill surface area. For comparison, the interlamellar channel width in the mako shark is roughly twice that found in tunas and thus the gill surface area in the mako shark is only half that found in the tuna (Weneger et al., 2010). This contributes to the reduced aerobic capacity reported for lamnids compared to tunas and to a reduction

in their sustainable swimming speeds (Bernal et al., 2003a,b; Sepulveda et al., 2007; Wegner et al., 2012).

Other features that have been proposed to contribute to the rigidity of the elasmobranch gill in ram-ventilating species are the corpus cavernosum in the gill filament, and vascular “sacs” found near the afferent (water entry) edge of lamellae in both the shortfin mako and blue shark, *Prionace glauca* (Cooke, 1980; De Vries and De Jager, 1984; Wegner et al., 2010). It has been proposed that the connection of both the corpora cavernosa and lamellar sacs to the respiratory circulation allows them to act as a hydrostatic skeleton, possibly subject to vasoactive control, that maintains both filament and lamellar structural integrity (Wegner et al., 2010). In the spiny dogfish (*Squalus acanthius*), “button-like epithelial outgrowths” have been described with the suggestion that they also act to keep the interlamellar spaces open (De Vries and De Jager, 1984). Finally, the thick epithelium on the leading lateral edges of mako and blue sharks, which resembles that described in the ram-ventilating teleost the wahoo (*Acanthocybium solandri*), may contribute to an overall bracing of the lamellae for ram ventilation (Wegner et al., 2006).

5. RELATIONSHIPS BETWEEN VENTILATION AND HEART RATE

The effectiveness of respiratory gas exchange over fish gills is optimized when the rates of countercurrent flow of blood and water are matched according to their relative capacities for oxygen (i.e., the capacity rate ratio = 1) (Piiper and Scheid, 1977). Because both flows are continuous, but pulsatile, this would require a functional relationship between the respiratory and cardiac rhythms that matches the maximum periods of flow in the two systems (Taylor, 1992). Typically, water flow is maximal early in the respiratory cycle and declines during the last two-thirds of the cycle (Hughes, 1960b, 1972; Hughes and Shelton, 1962). Recordings of differential blood pressure and gill opacity in the dogfish reveal a brief period of rapid blood flow through the lamellae, and because ventricular ejection (shown by electrocardiogram) tends to occur at or near the mouth-opening phase of the ventilatory cycle, there should be a coincidence of maximum flow rates of blood and water during each cardiac cycle (Satchell, 1960; Shelton, 1970).

Hughes (1972) explored evidence for phase coupling between ventilation and heartbeat in restrained catshark. Sophisticated analysis using event correlograms revealed that, in some cases, the heart did indeed tend to beat in a particular phase of the ventilatory cycle for short periods and use of polar coordinates revealed significant coupling at varied phase angles between the

two rhythms. However, experimentally restrained catsharks showed no hypoxic ventilatory response (Butler and Taylor, 1971) and no evidence of maintained cardiorespiratory synchrony (Hughes, 1972; Taylor and Butler, 1971). Ventilation rate was approximately twice heart rate and its relationship with heart rate drifted over time (Taylor and Butler, 1971; Taylor, 1985). In contrast, unrestrained catsharks showed reduced normoxic ventilation rates plus a ventilatory response to hypoxia (Metcalf and Butler, 1984) and synchrony between heartbeat and ventilation (Taylor, 1992). In unrestrained cod (*Gadus morhua*) heartbeat became associated with a particular phase of the respiratory cycle in moderate hypoxia (Taylor, 2011b). These observations suggest that maintenance of particular phase relationships between heartbeat and ventilation are dependent on the conditions under which these variables are measured and the consequent state of the fish. The absence of synchrony, or even consistent close coupling, between heartbeat and ventilation appears most often attributable to experimental restraint and a consequent higher variability in heart rate in relation to ventilation rate in catshark (Hughes, 1972; Taylor and Butler, 1971; Taylor, 1992).

Control of the heart is exercised by the autonomic nervous system that is well-developed in all jawed vertebrates, being clearly differentiated into parasympathetic and sympathetic components (Taylor, 1992; see also Chapter 1). However, the sympathetic nervous system does not extend into the cephalic and orobranchial regions in front of the pectoral girdle in elasmobranchs (Young, 1958). As a result, there is no direct sympathetic innervation of the heart or the branchial circulation to the gills. Nervous control of the heart, branchial blood flow, and cardiorespiratory interactions is exercised solely by parasympathetic, vagal innervation (Butler and Taylor, 1971; Taylor et al., 1977; Barrett and Taylor, 1984; Metcalf and Butler, 1984; Taylor, 1992), although circulating catecholamines have been shown to modulate the sensitivity of the heart to parasympathetic control (Agnisola et al., 2003). Because they lack sympathetic innervation to the heart, any regulated changes in heart rate reflect variations in cardiac vagal tone. The heart normally operates under a degree of inhibitory vagal tone that varies with temperature and oxygen level (Taylor, 1992). The critical oxygen level (P_{crit}) for onset of a reflex bradycardia during progressive hypoxia is directly related to experimental temperature, while progressive hyperoxia induces an initial increase in heart rate followed by a bradycardia that may arise from stimulation of venous oxygen receptors (Butler and Taylor, 1975; Taylor et al., 1977; Barrett and Taylor, 1984). A decrease in cardiac vagal tone, such as that recorded during exposure to moderately hyperoxic water, causes heart rate to rise toward ventilation rate. This suggests that when vagal tone is relatively low, a 1:1 synchrony could occur. When unrestrained catshark (cannulated for measurement of blood and

ventilatory pressures) are allowed to settle in large tanks of running aerated seawater at 23°C, they show 1:1 synchrony between heartbeat and ventilation during prolonged periods of inactivity that is lost on disturbance (Taylor, 1992).

All of these recorded changes in heart rate were abolished by injection of the muscarinic cholinceptor antagonist, atropine, which demonstrates that the heart in catsharks operates under chronic parasympathetic control (Taylor, 1992). Thus, the cardiac branch of the vagus nerve is continuously active. The nature of efferent outflow along the cardiac vagus is complex. Recordings from the central cut ends of branches of the cardiac vagus in decerebrate and paralyzed catshark show two different forms of activity; small units of activity that occur sporadically or continuously but without any clear pattern, and larger units of activity that burst in synchrony with respiration-related activity in branchial nerves (Figs. 2.5B and 2.11; Taylor and Butler, 1982; Barrett and Taylor, 1985a). As these forms of activity were recorded from paralyzed fish being ventilated continuously, with no phasic water flow, the bursting, respiration-related activity must have been generated centrally. Stimuli that cause a marked bradycardia, such as hypoxia or stimulation of J-receptors by capsaicin, increase activity in the sporadically active units and thus increase cardiac vagal tone (Fig. 2.12; Taylor and Butler, 1982; Taylor, 1992; Jones et al., 1993). The reflex hypoxic bradycardia recorded in catshark is likely to be attributable to increased activity in these units (Butler and Taylor, 1971; Taylor et al., 1977; Taylor and Butler, 1982). When vagal tone is relatively low (i.e., in settled normoxic

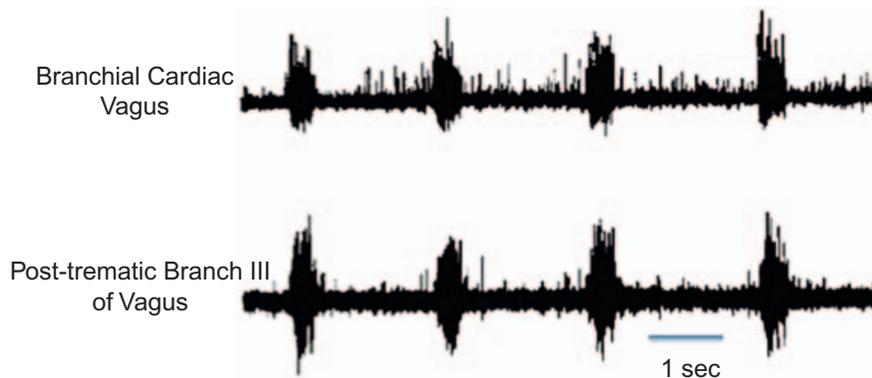


Figure 2.11. Rhythmic bursting of efferent activity synchronous with ventilatory movements recorded from the post-trematic 3rd branchial branch of the vagus and from the branchial cardiac branch of the vagus nerve in a decerebrated and paralyzed catshark. Note the relatively high rate of bursting in both nerves and the presence of sporadically firing units in the branchial cardiac nerve. From Barrett and Taylor (1985a, Fig. 2.1B), with permission.

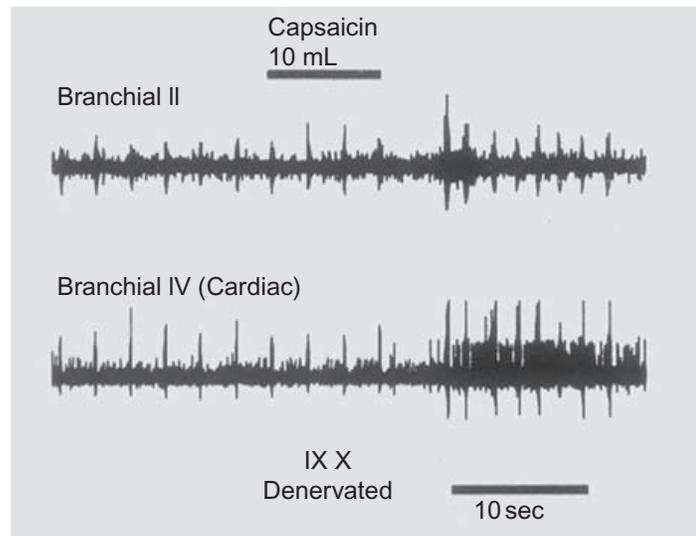


Figure 2.12. Recordings of efferent nervous activity in the second branchial (respiratory) branch (Branchial II) and the branchial cardiac branch [Branchial IV (Cardiac)] of the vagus nerve, in a decerebrated, paralyzed catshark, *S. canicula*. Injection of a bolus of dilute capsaicin (red hot chili pepper) into the water stream irrigating the gills caused a brief cardiac arrest accompanied by an increase in the rate of fictive ventilation (indicated by the rate of the bursting activity in the respiratory branch) and the onset of a prolonged burst of activity in nonbursting units in the cardiac nerve that are likely to have induced the bradycardia. From Jones et al. (1993), with permission.

or hyperoxic fish), firing rates in the sporadically active units are reduced, while the respiration-related activity in the larger units is unaffected (Taylor, 1992). This bursting activity may serve to modulate heart rate, which generates the cardiorespiratory synchrony reported from unrestrained catshark or those exposed to moderately hyperoxic water (Taylor, 1992).

A role for bursting efferent activity in the cardiac vagus in modulating heart rate (f_H) has been confirmed by electrical stimulation. Tonic stimulation of the peripheral cut end of a cardiac branch slows the heart. On the other hand, delivery of bursts of stimuli that simulate the respiration-related activity recorded centrally from the cardiac nerve entrain the heart over a wide frequency range that can be below or above the intrinsic, pre-stimulation rate (Young et al., 1993; Taylor et al., 2006). In a single example of a fish with an intrinsic f_H of 36 beats min^{-1} , f_H was driven at rates from 19 to 41 beats min^{-1} . When the burst frequency was increased to 43 beats min^{-1} , f_H was recruited at half this rate (Taylor et al., 2006), which implies the bursts are pacing the heart at rates below the refractory period.

The different types of activity recorded from the cardiac vagus originate from cardiac vagal preganglionic neurons (CVPNs), which have separate locations in the medulla (Barrett and Taylor, 1985b,c; Taylor, 1992). Those

showing respiration-related activity are located in the DVN of the vagus nerve, where they are co-located with neurons innervating respiratory muscles in the gill arches (Fig. 2.4B). This co-location could enable recruitment of CVPN by activity in neighboring respiratory vagal motor neurons (RVMN). In contrast, the sporadically active units show a scattered distribution ventro-lateral of the DVN, which removes them from possible recruitment (Fig. 2.4B). These observations indicate that cardiorespiratory synchrony (CRS) may be generated in the catshark by central, feed-forward control; either directly from the respiratory CRG or following recruitment by RVMN, when overall vagal tone on the heart is relatively low.

The situation described here in elasmobranchs is quite different than that in teleosts (Taylor et al., 2006, 2009b). In elasmobranchs, the cardiac vagus shows efferent bursting activity in normoxic, decerebrate, and paralyzed fish, implying centrally generated activity; while in teleosts the cardiac vagus is silent in normoxia with respiration-related, bursting activity appearing during progressive hypoxia together with the development of CRS (Leite et al., 2009). This is attributed to the stimulation of gill mechanoreceptors by increased ventilatory effort, supported by the fact that peripheral stimulation of cardiac vagi or central stimulation of branchial nerves in pacu (*Piaractus mesopotamicus*) have been shown to be capable of entraining heartbeat (Taylor et al., 2009a). Further support for this contention is provided by the observation that CRS could be generated in rainbow trout (*Oncorhynchus mykiss*) by pulsatile forced ventilation, independent of central respiratory rhythmicity (Randall and Smith, 1967). Thus, fundamentally different mechanisms may underlie the generation of CRS in teleosts and elasmobranchs (Taylor et al., 1999). As reported above, CRS was observed directly in catshark when they were allowed to settle unrestrained in large tanks of aerated seawater (Taylor, 1992). So it appears that CRS may be characteristic of settled fish with low levels of cardiac vagal tone, while exposure to hypoxia and a consequent increase in cardiac vagal tone may be necessary to induce CRS in teleosts (Taylor et al., 2006, 2009b; Leite et al., 2009).

However, physical stimulation of a gill septum or phasic central stimulation of a branchial branch of the vagus can drive efferent bursting activity in a cardiac branch in the catshark (Barrett and Taylor, 1985c; Young et al., 1993) and in the pacu (Taylor et al., 2009a). Therefore, peripheral feedback mechanisms exist in both elasmobranchs and teleosts, but with apparent differing emphases in their relative roles in the generation of CRS. In pacu phasic central stimulation of respiratory branches of the IXth and VIIth cranial nerves was also successful in recruiting the heart. However, central stimulation of the Vth cranial nerve was without effect on

heart rate, possibly reflecting its discrete role in cardiorespiratory control (Taylor et al., 2009a). Similar data is lacking for elasmobranch fishes and this invites further investigation.

Modulation of heart rate by respiratory activity can also be detected as a respiratory component in the heart rate variability (HRV) signal recorded by Power Spectral Analysis. To date, this technique has not been applied to an elasmobranch fish but it has been successfully used on teleosts (Altimiras et al., 1994, 1995, 1996). In the sculpin, *Myoxocephalus scorpius* a respiratory component was identified in the HRV signal after application of the Nyquist criterion and an antialiasing filter (Campbell et al., 2004, 2005). Reanalysis of their data (Taylor et al., 2006) indicated that establishment of CRS may be an important factor in optimizing the efficiency of oxygen uptake (maximum gain for minimum effort). Thus, the importance of a close relationship between heartbeat and ventilation in elasmobranch fishes merits further study using techniques of spectral analysis, including the coherence and phase of the variability between ventilation cycles and heart rate, in order to identify the mechanisms generating fine control of the cardiorespiratory system in these fish.

6. AFFERENT INPUT

6.1. Respiratory Reflexes

6.1.1. HYPOXIA

In elasmobranchs studied to date (mostly catshark), the response to hypoxia in individuals at rest is an increase in ventilation volume and decrease in heart rate, just as it is in teleosts (Perry et al., 2009a). The hyperventilation is modest, however, and any disturbance that raises resting ventilation seems to abolish it (Butler and Metcalfe, 1988). The hyperventilation is primarily due to increases in breathing amplitude rather than breathing frequency, a strategy that is energetically favorable for fish (Shelton et al., 1986; Perry and Wood, 1989; Gilmour, 2001). Rapidly induced hypoxia causes a marked reflex bradycardia in the catshark, followed by partial recovery to a rate determined by the level of hypoxic exposure. This response is mediated via the dual innervation of the heart by the cardiac vagus (Taylor et al., 1977). Although the reduction in heart rate is accompanied by an increase in cardiac stroke volume, this is only sufficient to maintain overall cardiac output. In contrast to most teleosts,

however, in elasmobranchs the fall in heart rate also decreases blood pressure, questioning their ability to show a baroreceptor response (Satchell, 1961; Butler and Taylor, 1971; Short et al., 1979; Taylor, 1992).

Increases in ventilation during progressive hypoxia, however, are insufficient to maintain the partial pressure of oxygen in arterialized blood (P_{aO_2}) as inspired P_{iO_2} declines (Butler and Taylor, 1975). Once the shoulder of the Hb- O_2 equilibrium curve is reached, blood oxygen content (CaO_2) also falls. At this point, oxygen consumption (MO_2) can only be maintained by an equivalent reduction in venous content (CvO_2). Ultimately, this is insufficient to maintain metabolic rate. The water PO_2 (PwO_2) at which the MO_2 of an organism begins to fall is termed the critical O_2 tension, or P_{crit} . At this point the fish transitions from oxyregulation to oxyconformation. A low P_{crit} is associated with greater hypoxia tolerance reflecting a greater ability to take up O_2 from the water and transport it to tissues to sustain metabolic rate. While physiological modifications at any step in the oxygen transport cascade can affect P_{crit} , differences in Hb- O_2 binding affinity have the greatest effect and there is a strong correlation between P_{crit} and the P_{50} of Hb (Dejours, 1981).

In general, elasmobranchs have a relatively high P_{crit} ; that is, their metabolic rates begin to fall quickly as hypoxia progresses as has been described for the bamboo shark (*Hemiscyllium plagiosum*; Chan and Wong, 1977). In catshark, MO_2 falls immediately on exposure to even modest hypoxia (Piiper et al., 1970), as it does in the pelagic blacktip reef shark (*Carcharhinus melanopterus*) and the shovelnose ray (*Rhinobatus typus*) (Routley et al., 2002). In the catshark, the response is temperature dependent. Environmental hypoxia has little effect at 7°C, but produces a fall in oxygen uptake at 12 and 17°C (Butler and Taylor, 1975).

A documented exception is the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*). It has a P_{crit} of 2.2 mg O_2 L^{-1} at 25°C, the lowest P_{crit} recorded for any elasmobranch (Routley et al., 2002). The epaulette shark lives on shallow reef platforms that surround islands of the Great Barrier Reef and Torres Strait (Last and Stevens, 1994). These areas experience repeated bouts of hypoxia during nocturnal low tides. The epaulette shark displays a biphasic ventilatory response to hypoxia, similar to that observed in teleosts and the catshark (Hughes, 1972; Ultsch et al., 1981). Exposure to hypoxia produces an initial increase in ventilation rate that serves to maintain MO_2 . In severe hypoxia, ventilation falls with a concomitant increase in the blood lactate level. This ventilatory depression is closely linked with the onset of a metabolic depression aimed at reducing ATP use in response to the reduction in aerobic ATP production (Routley et al., 2002) as is seen in many other vertebrates (Lutz and Nilsson, 1997).

Hypoxic preconditioning of the epaulette shark depresses MO_2 by 29% and lowers P_{crit} from 2.2 to 1.7 mg O₂ L⁻¹, further increasing its hypoxia tolerance (Stensløkken et al., 2004; Routley et al., 2002).

In the bonnethead shark (*Sphyrna tiburo*), an obligate ram ventilator, a fall in oxygen concentration to <4.5 mg L⁻¹ leads to an increase in swimming speed and mouth gape (Parsons and Carlson, 1998; Carlson and Parsons, 2001). During exposure to decreasing levels of dissolved oxygen, this species increases mouth gape from 0.8 cm at 6.0 mg L⁻¹ to 2.2 cm at 3.0 mg L⁻¹, while ventilation volume increases from 0.61 to 5.28 L min⁻¹ kg⁻¹. This increase in ventilation volume is sufficient to maintain blood oxygen content constant and to sustain MO_2 (Carlson and Parsons, 2003). Because increasing swimming speed is metabolically costly (i.e., oxygen consumption rate increases with speed as a power function), a point will be reached where oxygen demand will exceed delivery and this strategy will be insufficient to sustain MO_2 (Carlson and Parsons, 2001).

In catshark, circulating catecholamines increase in response to hypoxia (Butler et al., 1978, 1979, 1986). Injection of epinephrine or norepinephrine increases ventilation, plus efferent activity in branchial and hypobranchial nerves and in respiration-related activity in central neurons (Taylor and Wilson, 1989; Randall and Taylor, 1991; Taylor et al., 2006). However, in the spiny dogfish circulating catecholamines were unchanged on exposure to hypoxia and injection of catecholamines into the circulation during hypercapnia or hypoxia (simulating the levels of adrenaline and noradrenaline that were measured in the studies on catshark) failed to elicit hyperventilation or to modify any of the measured blood respiratory variables. Ventilation frequency was actually reduced significantly after catecholamine injection, not increased (Perry and Gilmour, 1996). These specific differences were mirrored in a study of modulation by the neurotransmitter neuropeptide Y of the effects of norepinephrine on the catshark (*S. canicula*) heart. A clear effect was described but wrongly ascribed to the dogfish, *S. acanthius* (Xiang et al., 1994). An unpublished follow-up study revealed that this effect was absent in the dogfish, which accords with it not releasing catecholamines in hypoxia. Thus, the dispute over a role for catecholamines in elevating ventilation in elasmobranchs may be based on species differences. This possibility merits correction and reconsideration.

6.1.2. HYPERCARBIA

The respiratory responses of fish to hypercapnia have been reviewed in detail previously (Gilmour, 2001; Perry and Gilmour, 2002; Gilmour and Perry, 2007). Although relatively few species have been studied, hyperventilation is a common response to elevated ambient PCO_2 (Gilmour, 2001; Perry and Gilmour, 2002; Gilmour and Perry, 2007).

Indeed, hyperventilatory responses have been observed in representatives of all the major fish groups including hagfish (Perry et al., 2009b); elasmobranchs (Graham et al., 1990; Heisler et al., 1988; McKendry et al., 2001; Randall et al., 1976); sturgeons (*Acipenser transmontanus*) (Crocker et al., 2000); gar (*Lepisosteus osteus*) (Smatresk and Cameron, 1982); lungfish (*Protopterus dolloi*, *Lepidosiren paradoxa*) (Perry et al., 2008; Sanchez et al., 2005; Soncini and Glass, 2000); and teleosts (Burlinson and Smatresk, 2000; Gilmour et al., 2005; Janssen and Randall, 1975; Perry and McKendry, 2001; Perry and Reid, 2002; Reid et al., 2000; Soncini and Glass, 2000; Sundin et al., 2000). The pronounced increase in ventilation volume in hypercapnic hagfish (Perry et al., 2009b) attests to the early evolutionary origin of CO₂-mediated respiratory drive.

Exposure to acute hypercarbia leads to an increase in ventilation in the dogfish, due primarily to increases in ventilatory amplitude and modest increases in breathing frequency (Randall et al., 1976; Graham et al., 1990; Perry and Gilmour, 1996; McKendry et al., 2001). It also produces a bradycardia, which leads to a reduction in cardiac output and lower arterial (post-branchial) blood pressure (McKendry and Perry, 2001). Since systemic vascular resistance is unaltered, this suggests that the hypotension is caused by the lowering of cardiac output, although, it has also been suggested that increased gill vascular resistance contributes to the overall hypotension (Kent and Peirce, 1978). Again as in hypoxia, this is in contrast to the hypertensive response seen in teleosts. The response in teleosts results from an increase in systemic vascular resistance caused by vasoconstriction following sympathetic activation of vascular smooth muscle α -adrenoreceptors (Perry et al., 1999a), a response that appears to be absent in elasmobranchs, possibly accounting for their failure to show a baroreceptor response to changes in blood volume (Taylor, 1992).

6.1.3. AMMONIA

In many fishes, including elasmobranchs, both environmental and plasma levels of ammonia may act as respiratory stimuli. Exposure to high environmental ammonia (HEA) in dogfish elicits a slowly developing hyperventilation with a time course that suggests the increase is due to increases in plasma ammonia content ($[T_{\text{Amm}}]$), thus indicating that the chemosensing of ammonia is internal (De Boeck and Wood, 2014). This may seem surprising given that dogfish are ureotelic and normally excrete only minimal amounts of ammonia (Wood et al., 1995). But there are several instances when plasma ammonia levels rise in dogfish, specifically post-feeding and post-exercise (Richards et al., 2003; Wood et al., 2005; Kajimura et al., 2008). Attempts to demonstrate that plasma $[T_{\text{Amm}}]$ can act directly as a respiratory stimulant have been confounded by the changes in acid–base status associated with injections of isotonic solutions of

(NH₄)₂SO₄ or NH₄HCO₃ in spiny dogfish, but certainly support the suggestion that this is the case (De Boeck and Wood, 2014). As with hypoxia and hypercarbia, the relative increases in ventilatory amplitude are much greater than those in frequency.

The ventilatory responses of dogfish to hypoxia, hypercarbia, and ammonia are essentially identical. This raises the question of whether the physiological responses are mediated by a single group of receptors, or by distinct receptor groups whose integrated output is directed to similar efferent pathways.

6.2. Receptors Involved in Reflex Ventilatory Control

There has been increased interest in identifying the receptors involved in producing ventilatory responses to respiratory gases in fish, along with their locations, orientation, and input pathways (Perry and Abdallah, 2012; Porteus et al., 2012; Zachar and Jonz, 2012). Most of the work has been done on teleosts, however, and the data that exist for elasmobranchs come exclusively from studies on dogfish.

Although not universal, the receptors involved in eliciting changes in heart rate and breathing frequency in response to hypoxia, hypercarbia, and ammonia in teleosts tend to be restricted to the gills. In contrast, those producing increases in breathing amplitude are more widespread, frequently also being found at extrabranchial sites. The distribution of the receptors in the gills sensitive to CO₂, and those involved in producing ventilatory responses, tend to be more restricted than the O₂-sensitive receptors. The specific location of the CO₂-sensitive receptors involved in the various components of the cardiorespiratory responses can vary from those of the O₂-sensitive receptors (Milsom, 2012).

In dogfish, the O₂-sensitive receptors eliciting bradycardia are not confined to the gills, but are also located throughout the orobranchial cavity, and are innervated by trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) nerves (Fig. 2.13; Butler et al., 1977). Similar studies on O₂-sensitive receptors eliciting ventilatory and hypotensive responses to hypoxia have not been performed.

All cardiorespiratory responses to hypercarbia, however, come exclusively from the gills in dogfish, and these responses are (i) eliminated by denervation of the branchial branches of glossopharyngeal (IXth) and vagal (Xth) nerves (Fig. 2.13; McKendry et al., 2001) and (ii) arise from the stimulation of externally oriented (water sensing) receptors (Gilmour et al., 1997, 2001; Perry and McKendry, 2001; McKendry and Perry, 2001; McKendry et al., 2001; Gilmour and Perry, 2007). Selective denervation of individual gill arches has not been performed so the relative distribution of

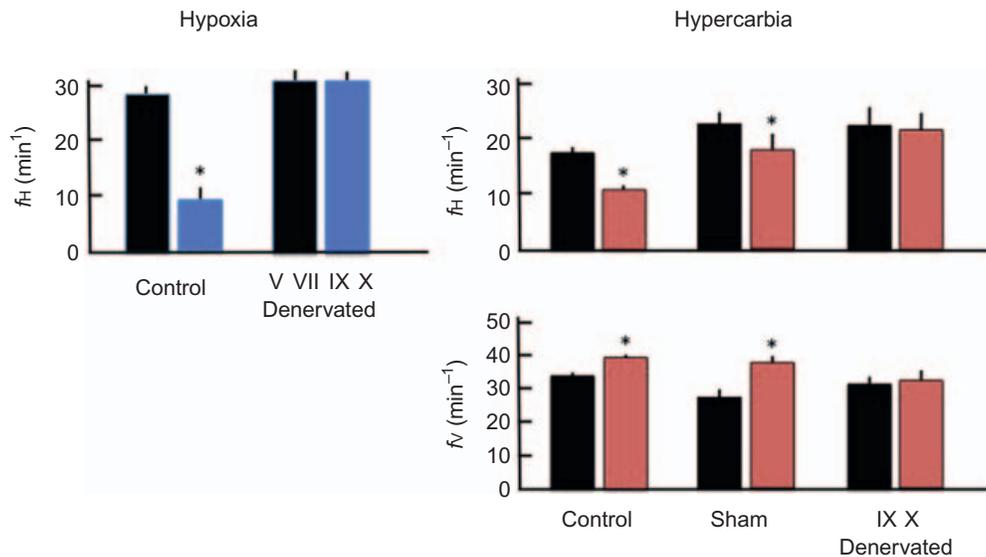


Figure 2.13. Left: The effects of external hypoxia (control, 16 mmHg; denervated, 6 mmHg) on heart rate (f_H). Right: The effects of external hypercarbia (control, 6.38 ± 0.13 mmHg; sham-operated, 6.58 ± 0.44 mmHg; denervated, 5.99 ± 0.54 mmHg) on heart rate (f_H) and ventilation frequency (f_V). Black columns represent normoxic, normocarbic values; blue columns represent maximal responses obtained during hypoxia and red bars represent maximal responses obtained during hypercarbia. Control fish were untreated, sham-operated fish underwent an identical procedure to the denervated fish except for the actual transection of cranial nerves, and denervated dogfish underwent surgical transection of all branchial branches of cranial nerves IX and X (hypercarbia) and cranial nerves V, VII, IX, and X (hypoxia). Values are means \pm 1 S.E. M. An asterisk denotes a significant difference between normoxia and hypoxia or normocarbica and hypercarbia ($P < 0.05$). Hypoxia data adapted from [Butler et al. \(1977\)](#); hypercarbia data adapted from [McKendry et al. \(2001\)](#).

the branchial CO_2 -sensitive receptors in dogfish associated with each component of the cardiorespiratory response (bradycardia, hypotension, and increase in breathing frequency and amplitude) has not been determined. Indirect evidence, however, suggests that CO_2 rather than H^+ acts specifically as the stimulus modality (although increasing H^+ may be the proximate intracellular stimulus) ([McKendry et al., 2001](#); [Perry and Abdallah, 2012](#)).

These observations would seem to rule out the existence of central CO_2 -sensitive receptors in dogfish ([Gilmour, 2001](#); [Perry and Abdallah, 2012](#); [Milsom, 2012](#)). There are, however, some data to implicate an internal site, as changes in ventilation in skate (*Raja ocellata*) during 24 h of aquatic hypercapnia are closely related to changes in arterial pH ([Graham et al., 1990](#); [Wood et al., 1990](#)).

Prior administration of the muscarinic receptor antagonist, atropine, abolishes the hypercarbia-induced ventilatory responses and virtually eliminates all CO_2 -elicited cardiovascular adjustments ([McKendry et al., 2001](#)).

This suggests that CO₂ chemoreception, or one or more downstream steps in the reflex pathway, or both, involve cholinergic (muscarinic) neurotransmission. Atropine administration also eliminates the hyperventilatory responses to external cyanide, which suggests a crucial role for muscarinic receptors in the response of dogfish to hypoxia as well. The latter is consistent with results obtained in rainbow trout suggesting that similar mechanisms exist in elasmobranchs and teleosts (Burlison and Milsom, 1995).

By contrast, increases in ventilation appear to only arise from stimulation by increasing levels of internal ammonia in elasmobranchs. In trout the response involves peripheral chemoreceptors in the 1st (primarily) and 2nd gill arches and there is strong circumstantial evidence that central chemoreceptors in the brain are also involved (Zhang et al., 2014). Studies have yet to be performed on an elasmobranch.

The receptors in the gills and orobranchial cavity believed to be involved in producing these responses are the serotonin-containing neuroepithelial cells (NECs) (Zachar and Jonz, 2012; Porteus et al., 2012; Perry and Abdallah, 2012). There is evidence that some, but not all, gill NECs are multimodal sensors potentially sensing O₂, CO₂, and NH₃ (Qin et al., 2010; Perry and Abdallah, 2012). Like O₂ sensing glomus cells in mammals, the mechanisms underlying the responses of NECs involve the inhibition of a background K⁺ current, which leads to membrane depolarization and subsequent elevation of intracellular Ca²⁺ levels (Zachar and Jonz, 2012). In teleosts, it has been suggested that an inwardly rectifying K⁺ (Kir) channel is a candidate for transducing O₂ and CO₂ levels (Perry and Abdallah, 2012). The CO₂ sensing appears to involve NECs specifically containing carbonic anhydrase. Catalyzing the hydration reaction of CO₂ to H⁺ and HCO₃⁻, appears to play a critical role in reducing NEC response times and increasing the magnitude of membrane depolarization accompanying hypercapnia. In larval zebrafish *in vivo*, carbonic anhydrase activity is essential for the rapid development of hypercapnic bradycardia (Perry and Abdallah, 2012). Finally, while there is evidence that CO₂ and ammonia may have a common signaling pathway in some fish NECs (Qin et al., 2010), it is also possible that ammonia enters fish gill NECs via Rh proteins producing a more immediate response (Zhang et al., 2014).

Centrally, the hyperventilatory responses to ammonia correlate more closely with concentrations of ammonia in the brain than those in plasma or cerebrospinal fluid. After chronic exposure to HEA, ventilatory responsiveness to ammonia is lost, and this is associated with both an attenuation of the [Ca²⁺]_i response in NECs, and the absence of elevation in brain ammonia concentration. Chronic exposure to HEA also increases the mRNA expression of several Rh proteins (ammonia-conductive channels) in both brain and gills (Zhang et al., 2014).

7. CONCLUSIONS

Compared with teleosts, elasmobranchs have been relatively neglected in studies by physiologists. This is an important oversight because this ancient group of vertebrates provides unique insights into the evolution of physiological processes common to all vertebrates, and in practical terms, they lend themselves well to the study of central integration of respiratory and cardiovascular systems because of their relatively large brains and cartilaginous cranium (Taylor, 1989, 2011a,b; Taylor et al., 2009b). Elasmobranchs also show a remarkable suite of adaptations to active predatory as well as relatively sedentary, benthic lifestyles. They are widely distributed in the world's oceans although, due to aggressive fishing practices and anthropogenic changes in their environment, their future is threatened. Our review has focused on the respiratory physiology of elasmobranchs, and concentrated on the generation of the respiratory rhythm, the patterns of motor output, the sources of key sensory inputs, and the functional links between respiration and the cardiovascular system that together are responsible for the exchange and transport of respiratory gases. Respiratory gas exchange has been much studied in teleost fishes and it has been established that a functional countercurrent flow of water and blood operates over the exchange surfaces of the gill lamellae. This generates a highly effective exchange of oxygen such that arterial blood can have a higher oxygen partial pressure than expired water. The structure of elasmobranch gills, however, seems to preclude this highly effective exchange of gases, as countercurrent flow over the gill lamellae is interrupted by the interbranchial septum and this may be countered by wider spacing of gill lamellae. In addition, the mechanisms generating the continuous (but pulsatile) flow of water over the exchange surfaces in elasmobranchs are different and apparently more variable than those described in the teleosts, with prolonged periods of reversed pressure or even flow recorded across the gills. Elasmobranchs lack an operculum, relying instead on separate parabranchial chambers to generate a suction pump. This offers a unique set of respiratory routes that include the spiracles and separate gill slits functioning independently, enabling ventilation to continue during prey capture and ingestion. Inspiration is passive in routinely active animals relying on elastic recoil in the pharyngeal skeleton. However, when activity levels are raised or oxygen supply is limiting, elasmobranchs can recruit feeding muscles innervated by hypobranchial nerves into the respiratory cycle thus generating forced inspiration.

The location of the respiratory rhythm generator in elasmobranchs remains as yet uncertain. The trigeminal nucleus and activity in the

trigeminal (Vth) nerve seem to initiate a pattern of activity that is transmitted rostrally-caudally through the sequential series of motor nuclei supplying other cranial nerves that innervate respiratory structures. This activity may be relayed to anterior spinal nerves contributing to the hypobranchial nerve complex and innervating feeding muscles that may be recruited into the respiratory cycle during forced ventilation. This rostrally-caudal spread of excitation follows the segmental origins of cranial nerves as reflected in their development and evolution. Respiration-related activity was recorded from neurons in the brainstem of fishes between 50 and 30 years ago but even now the central origins of respiratory rhythm generation (RRG) remain unclear. Consequently we have constructed a paradigm for RRG in elasmobranchs, on the basis of studies on neonates and cyclosoemes, that presents a null hypothesis inviting critical investigation. Elucidation of the mechanisms generating respiratory activity will require more careful study of the central projections to the motor nuclei and their interrelationships in the brainstem (Figs. 2.1, 2.3, 2.4A,B, and 2.14). One route in this exploration could be the central injection of neural tracers to trace pathways that project to the respiratory motor nuclei and in particular to the Vth, trigeminal nuclei.

Effective exchange of respiratory gases across the gills is dependent on an optimal relationship between capacity rate ratio and the patterns of water and blood flow over the exchange surfaces of the gill lamellae. Both are affected by exposure to hypoxia or elevated levels of CO₂ or ammonia, with receptors for each of these gases located on the gills or more diffusely over the extrabranchial surfaces of the respiratory chambers and possibly in the CNS. Although there are apparent differences in the distribution of receptors to each of these gases, there is also some evidence that NECs on the gills show responses to all three. The transduction of information from receptors for oxygen and CO₂ may involve muscarinic receptors and this possibility may have affected studies of a functional role for hypoxic bradycardia based on the systemic injection of atropine. As both blood and water flows are highly pulsatile, albeit continuous, it has long been hypothesized that respiratory gas exchange could be optimized by temporal relationships between the respiratory and cardiac pumps. Periods of simultaneous occurrence of maximum blood and water flow rates over the gills have been recorded in sharks and respiration-related efferent bursting activity has been recorded from the cardiac branches of the vagus nerve. This is generated centrally so that the heart is subject to feed-forward control. In the spontaneously breathing fish, respiration-related efferent activity can also be generated reflexively by stimulation of gill mechanoreceptors. When this bursting activity was generated by electrical stimulation it paced the heart at rates both above and below the intrinsic

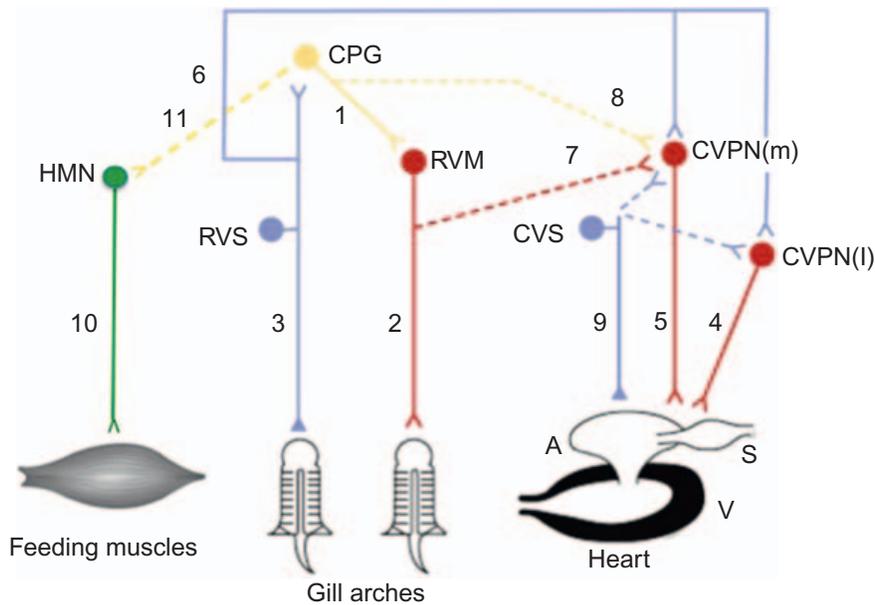


Figure 2.14. A schematic model of possible connections in the central and peripheral nervous systems generating the respiratory rhythm, cardiorespiratory interactions, and recruitment of feeding muscles in elasmobranch fishes. There are several established connections: (1) a respiratory central pattern generator (CPG) shows endogenous bursting activity that drives respiratory motor neurons (RVM); (2) the RVMs innervate respiratory muscles via the V, VII, IX, and X cranial nerves; (3) the activity of the CPG is modulated by feedback from mechanoreceptors and chemoreceptors located in the orobranchial cavity, innervated by sensory neurons (RVS). Heart rate is controlled by inhibitory input from the vagus nerve that receives axons from CVPNs, which are topographically and functionally separable into (4) a ventro-lateral group [CVPN (l)], some of which fire continuously and others that fire in response to hypoxia or mechanical stimulation and may be responsible for reflex changes in heart rate (e.g., hypoxic bradycardia) and for the varying level of vagal tone on the heart, and (5) a medial group [CVPN (m)], which burst with a respiration-related rhythm and may cause the heart to beat in phase with ventilation. (6) Stimulation of receptors in the orobranchial cavity, innervated by the VII, IX, and X cranial nerves but apparently not by the Vth, directly modify activity in both groups of CVPN. Other more speculative connections may determine the activity in CVPN: (7) collateral's from neighboring RVM may have an excitatory effect on bursting CVPN (m) (or release a tonic inhibition); (8) the CPG may connect directly to CVPN (m); (9) stimulation of receptors in the cardiovascular system close to the heart, innervated by vagal sensory neurons (CVS) may affect vagal outflow to the heart. Feeding muscles are innervated by the hypobranchial nerves (HMN) (10) and can be recruited into the respiratory cycle, possibly by direct inputs from the CPG (11) or from neighboring RVM (link not shown but see Fig. 2.4B). This diagram ignores the existence and possible roles of interneurons and inputs from and to higher centers in the CNS. A, atrium; CVPN, cardiac vagal preganglionic neuron; CVS, cardiac sensory neuron; HMN, hypobranchial motor neuron; RVS, respiratory sensory neuron; S, sinus venosus; V, ventricle. Inverted V, efferent termination; filled triangle, afferent termination. Modified from Taylor (1992).

rate, which implies that the vagus nerve does not merely operate by imposing a chronic inhibitory tonus. The cardiac branch of the vagus (Xth) nerve also transmits nonbursting efferent activity that increases during hypoxia and on stimulation of J-receptors, which is likely responsible for the observed bradycardia. This separation of function relates to the location of CVPNs in the brainstem and this anatomical and functional separation reflects that observed in the mammalian brain (Taylor et al., 1999; Grossman and Taylor, 2007). Elasmobranchs lack sympathetic innervation of the heart but norepinephrine modulates vagal control of the heart and there remains a debate over a role for circulating catecholamines in control of ventilation. The dependence of cardiac chronotropic control on both chronic and phasic vagal efferent activity transmitted via muscarinic receptors identifies elasmobranchs as a model for study of the instantaneous control of heartbeat and its relation with ventilation by the parasympathetic nervous system, operating via the cardiac branch of vagus (Xth) nerve in all vertebrates. The possible functional connections generating these interactions in elasmobranchs are summarized in Fig. 2.14.

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OXYGEN AND CARBON DIOXIDE TRANSPORT IN ELASMOBRANCHS

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The elasmobranchs are an ecologically diverse subclass of over 1000 species that have evolved to inhabit a wide range of environments and become one of the most speciose groups of vertebrate predators on Earth. This chapter reviews what is known about elasmobranch O₂ uptake, transport, and delivery, as well as CO₂ transport and elimination, and focuses upon two metalloproteins central to these processes, hemoglobin (Hb) and carbonic anhydrase (CA), both of which have undergone distinct functional adaptations. Furthermore, adaptations in relation to life history, which include exercise, hypoxia, salinity, temperature, and, in some species, regional heterothermy, are reviewed. While processes and principles of gas transport and exchange in elasmobranchs are often similar to those of the better described teleosts, there are differences that stand out as clearly worthy of further investigation. Generally, elasmobranch Hbs exhibit a high affinity for O₂ relative to teleosts, which may be associated with a low organic phosphate/Hb ratio and an antagonistic effect of urea on Hb-ATP sensitivity. The Hbs also exhibit a moderate Bohr and Haldane effect, but high buffering by Hb and plasma proteins coupled with the presence of

plasma accessible CA greatly reduces the interaction between O₂ and CO₂ exchange relative to the situation in teleosts. Moreover, at least in the dogfish, *Squalus suckleyi*, current models of CO₂ excretion suggest similar contributions of the plasma and red blood cell (RBC) to CO₂ excretion, a model that contrasts with the pattern of CO₂ excretion typical of other vertebrates in which near exclusive reliance is placed on the RBC. High plasma buffering and plasma-accessible CA in the gill of dogfish favor HCO₃⁻ dehydration in the plasma, while HCO₃⁻ dehydration via the RBC is constrained by low RBC CA activity and the absence of a Haldane effect in this species. In the Hb of the whip stingray, *Dasyatis akajei*, a novel Bohr effect mechanism has been discovered and this same species possesses a novel ATP binding site in Hb. Finally, in the high performing regionally heterothermic sharks, there appears to be a reduction or reversal in Hb temperature sensitivity consistent with regionally heterothermic teleosts, but this remains to be investigated in detail. While gas transport and exchange is a central process associated with the success of elasmobranchs, it has been most thoroughly investigated in just a few species; clearly a great deal remains to be discovered to achieve a more representative understanding of gas transport and exchange in elasmobranch fish.

1. INTRODUCTION

Gas exchange is a prerequisite for aerobic life. In vertebrates the uptake of environmental oxygen (O₂) and the elimination of metabolic carbon dioxide (CO₂) require a gas exchange organ and a system to transport respiratory gases and acid–base equivalents between their sites of consumption or production and the gas exchange organ. Gas transport in vertebrates is achieved through a closed circulatory system; indeed, the basic vertebrate respiratory and circulatory systems were inherited by all extant jawed vertebrates from the most recent common ancestor to both the Osteichthyan and Chondrichthyan fishes, and these systems have been reviewed extensively (e.g., [Randall, 1970a,b](#); [Butler and Metcalfe, 1988](#); [Bushnell et al., 1992](#); [Satchell, 1992, 1999](#); [Perry and Tufts, 1998](#); [Brauner and Berenbrink, 2007](#); [Farrell, 2007](#); see also Chapter 1). Gas exchange in the adults of all known extant Chondrichthyan fishes primarily occurs across five to seven paired filamentous gill arches ([Hughes, 1984](#); [Butler and Metcalfe, 1988](#); [Butler, 1999](#); [Wegner, 2015](#)). Respiratory gases are transported in the blood, around the circulatory system, pumped by the heart. Central to the transport and exchange of respiratory gases in vertebrates are two metalloproteins, hemoglobin (Hb) and carbonic anhydrase (CA), which have undergone distinct functional adaptations

within the elasmobranchs to enhance O₂ delivery and accelerate CO₂ elimination respectively.

Elasmobranchs (Selachimorpha and Batoidea) and holocephalans (Chimaeriformes) comprise all extant representatives in the class Chondrichthyes (Janvier and Pradel, 2015). The elasmobranchs are an ecologically diverse subclass of fishes that have evolved to inhabit a wide range of environments and become one of the most speciose groups of vertebrate predators on Earth (Compagno, 1990; Dulvy et al., 2014; Janvier and Pradel, 2015). The osmoconforming strategy of elasmobranchs (see Chapter 4; Ballantyne and Fraser, 2013) and selective forces imposed by the diversity of environments that they inhabit influence gas transport and exchange. Therefore, comparative physiological investigations between the Osteichthyan and Chondrichthyan fishes provide insight into how components of the respiratory cascade have been modified to suit different species that share a similar habitat or lifestyle, but have evolved different osmoregulatory strategies and are separated by over 400 million years of evolutionary history (Janvier, 2007; Janvier and Pradel, 2015). However, there is still much to be learned about gas transport and exchange in the elasmobranchs. Investigations into the respiratory physiology of elasmobranchs date back to the late 19th and early 20th centuries (see Hyde, 1908; Piiper et al., 1970), with much of the knowledge having been collected from experiments on a few small, sedentary species that are relatively easy to catch and maintain in laboratory aquaria (e.g., *Squalus acanthias*, *Squalus suckleyi*, *Scyliorhinus canicula*, *Scyliorhinus stellaris*, *Heterodontus portus-jacksoni*, *Hemiscyllium ocellatum*, and *Leocoraja erinacea*). Because of their similar morphology and ecology, comparative physiologists tend to lump a few of these and other species into a group referred to as “dogfish,” but it is important to remember that these sharks are separated by as much as 300 hundred million years of evolutionary history (Grogan et al., 2012; Sorenson et al., 2014; Janvier and Pradel, 2015). Table 3.1 lists these “dogfish” sharks as well as the most commonly discussed species in this review along with synonyms and misapplied names. Much of the early work in the field has been well reviewed (see Randall, 1970a; Hughes, 1984; Piiper and Scheid, 1984; Randall and Daxboeck, 1984; Butler and Metcalfe, 1988; Nikinmaa and Salama, 1998; Tufts and Perry, 1998; Butler, 1999; Gilmour and Perry, 2010). However, the only reviews dedicated exclusively to elasmobranch respiratory physiology are those by Butler and Metcalfe (1988) and Butler (1999), which primarily covered the cardiorespiratory system with a strong focus on the anatomy of the gill and the cardiovascular system. Here we provide a thorough overview of Hb, CA, and red blood cell (RBC) function in gas transport and exchange in elasmobranchs, along with analyses of O₂ and CO₂ transport in the blood.

Table 3.1
Elasmobranchs species commonly discussed in this review

| Species | Synonyms | Environment and distribution |
|--|---|--|
| Skates | | |
| Arctic skate, <i>Amblyraja hyperborea</i> | <i>Raja hyperborea</i> | Bathydemersal, temperate to polar, northern and southern hemispheres |
| Eaton's skate, <i>Bathyraja eatonii</i> | <i>Raja eatonii</i> | Demersal, polar, Southern Ocean, southeast Pacific |
| Little skate, <i>Leucoraja erinacea</i> | <i>Raja erinacea</i> | Demersal, temperate, west Atlantic |
| Winter skate, <i>Leucoraja ocellata</i> | <i>Raja ocellata</i> | Demersal, temperate, west Atlantic |
| Myliobatid rays | | |
| Bat eagle ray, <i>Myliobatis californica</i> | | Demersal, temperate to sub-tropical, east Pacific |
| Cownose ray, <i>Rhinoptera bonasus</i> | <i>R. quadriloba</i> | Benthopelagic, temperate to tropical, west and east Atlantic |
| Whip stingray, <i>Dasyatis akajei</i> | Japanese stingray, red stingray, <i>Trygon akajei</i> | Demersal, temperate to tropical, west Pacific |
| Atlantic stingray, <i>Dasyatis sabina</i> | | Demersal, coastal and inshore, euryhaline, temperate to sub-tropical, western Atlantic |
| South American freshwater stingray, <i>Potamotrygon motoro</i> | Amazonian freshwater stingray, <i>P. circularis</i> , <i>P. laticep</i> | Benthopelagic, tropical, freshwater, South America |
| Sharks | | |
| Bull shark, <i>Carcharhinus leucas</i> | <i>C. nicaraguensis</i> , <i>C. zambezensis</i> | Coastal and inshore, euryhaline, tropical to sub-tropical, world-wide |
| Sandbar shark, <i>Carcharhinus plumbeus</i> | Brown shark, <i>C. milberti</i> , <i>Eulamia milberti</i> | Coastal and pelagic, temperate to sub-tropical, cosmopolitan |
| Lemon shark, <i>Negaprion brevirostris</i> | <i>Hypoprion brevirostris</i> | Inshore and coastal, tropical to sub-tropical, west Atlantic, northeast Atlantic, east Pacific |
| Leopard shark, <i>Triakis semifasciata</i> | | Demersal, temperate to sub-tropical, northeast Pacific |

| | | |
|---|---|---|
| Draughtsboard shark, <i>Cephaloscyllium isabellum</i> | Carpet shark, <i>C. isabella</i> | Demersal, subtropical, southwest Pacific (New Zealand) |
| Port Jackson shark, <i>Heterodontus portusjacksoni</i> | Bull or horn shark, <i>Squalus portusjacksoni</i> | Demersal, temperate to subtropical, west Pacific (Australia) |
| Epaulette shark, <i>Hemiscyllium ocellatum</i> | <i>Squalus ocellatus</i> | Demersal, reef-associated, tropical, southwest Pacific |
| <hr/> | | |
| “Dogfish” sharks | | |
| Spiny dogfish, <i>Squalus acanthias</i> | Piked or piked dogfish, Pacific dogfish, spurdog, rock salmon, <i>S. suckleyi</i> | Benthopelagic, marine and brackish, temperate, north Atlantic and southern hemisphere |
| Pacific spiny dogfish, <i>Squalus suckleyi</i> ^a | Spotted spiny dogfish, piked dogfish, <i>S. acanthias</i> | Benthopelagic, marine and brackish, temperate, north Pacific |
| Small-spotted catshark, <i>Scyliorhinus canicula</i> | Lesser-spotted dogfish, <i>Squalus canicula</i> | Demersal, temperate to sub-tropical, northeast Atlantic |
| Nursehound, <i>Scyliorhinus stellaris</i> | Greater- or larger-spotted dogfish, European dogfish, <i>Squalus stellaris</i> | Demersal, temperate to sub-tropical, northeast Atlantic |
| Dusky smooth-hound, <i>Mustelus canis</i> | Smooth dogfish | Demersal, marine and brackish, temperate to sub-tropical, west Atlantic |
| Spotless smooth-hound, <i>Mustelus griseus</i> | Japanese smooth-hound, smooth dogfish | Demersal, temperate to sub-tropical, northwest Pacific |
| <hr/> | | |
| Lamnid sharks (regional heterotherms) | | |
| Shortfin mako, <i>Isurus oxyrinchus</i> | Mako, blue pointer | Pelagic and coastal, temperate to subtropical, cosmopolitan |
| Salmon shark, <i>Lamna ditropis</i> | Mackerel shark, Pacific Porbeagle, <i>L. ditropis</i> | Pelagic and coastal, temperate, north Pacific |
| Porbeagle shark, <i>Lamna nasus</i> | Mackerel shark, <i>L. cornubica</i> | Pelagic and coastal, temperate, north Atlantic and southern hemisphere |

Current nomenclature, and distribution information were taken from FishBase (Froese and Pauly, 2011), and Compagno et al. (2005).

^aThe Pacific spotted spiny dogfish, formerly considered *Squalus acanthias*, has been reclassified as *Squalus suckleyi* based on life history and genetic differences (Ebert et al., 2010; Verissimo et al., 2010). The separation of these two species is further supported by sequence differences in their Na⁺/H⁺-exchangers (i.e., NHE2), indicating the possibility of physiological differences between *Squalus acanthias* and *Squalus suckleyi* (Guffey, 2013). Accordingly, any dogfish identified in the literature as *Squalus acanthias*, but caught in the north Pacific we have considered as *Squalus suckleyi*.

2. BLOOD-OXYGEN TRANSPORT

Aspects of the blood-O₂ transport characteristics of elasmobranchs were covered in a previous volume of the *Fish Physiology* series (Brauner and Randall, 1998; Gallagher and Farrell, 1998; Jensen et al., 1998; Nikinmaa and Salama, 1998). Although elasmobranchs were included in the chapters of that volume, the general focus was on teleosts. The diffusion of environmental O₂ across the gills into the blood of elasmobranchs is reviewed by Wegner (2015). The majority of O₂ that diffuses across the gills into the blood binds reversibly to Hb, encapsulated within the RBCs, and is then convectively transported by the actions of the heart throughout the circulatory system. The cardiovascular and circulatory systems of elasmobranchs are reviewed by Brill and Lai (Chapter 1). In the tissue capillary beds, the partial pressure of O₂ (*PO*₂) in the blood is higher than the *PO*₂ of the metabolically active tissues owing to the steady consumption of O₂, and this difference provides the driving force for O₂ diffusion from the blood into the tissues. Hb-O₂ transport can be “fine-tuned” in response to environmental and metabolic demands by interspecific and intraspecific increases in Hb concentration or through modulating Hb-O₂ binding characteristics (Wells, 1999). Thus, the characteristics of Hb and its microenvironment within the RBCs dictate the nature of blood-O₂ transport (e.g., Nikinmaa, 1997; Nikinmaa and Salama, 1998; Brauner and Val, 2005; Wells, 2005; Brauner and Berenbrink, 2007).

2.1. Hemoglobin

Hemoglobin has been superbly shaped by evolution to fulfill the job of binding and transporting O₂ from the gas exchange organ to the metabolically active tissues. Globins or the genes that code for them have been found in all kingdoms, which indicates their importance for physiological function (Weber and Vinogradov, 2001). Within the jawed vertebrates the tetrameric structure of Hb is highly conserved, but primary structural differences underlie functional adaptations to modulate O₂ binding affinity in response to internal and external environmental conditions (Weber and Fago, 2004). Hb structure and function in fishes has been reviewed in previous volumes of the *Fish Physiology* series (Riggs, 1970; Jensen et al., 1998), but much has been learned about elasmobranch Hbs more recently. It has been generally accepted that the Hbs of elasmobranchs lacked the functional adaptations typical of teleosts that allow fine-tuning of Hb-O₂ affinity according to environmental and metabolic demands (Hall and McCutcheon, 1938; Bonaventura et al., 1974a;

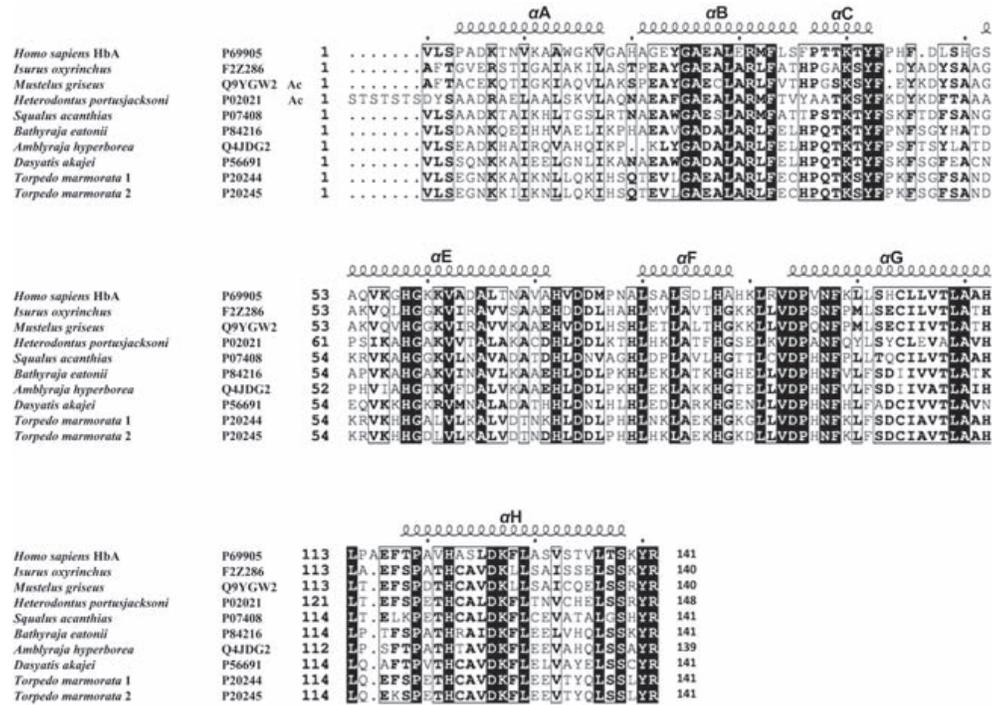
Pennelly et al., 1975; Dickinson and Gibson, 1981; Brittain et al., 1982; Gibson and Carey, 1982; Weber et al., 1983a; Wells et al., 1992; Wells, 1999). Although elasmobranch Hbs do not exhibit the extreme pH sensitivity that is characteristic of teleosts (Pennelly et al., 1975; Farmer et al., 1979; Ingermann and Terwilliger, 1982; Dafré and Wilhelm, 1989; Pelster and Weber, 1990; Berenbrink et al., 2005), there are functional similarities between elasmobranch Hbs and those of other vertebrate lineages that have arisen through distinct mutations that allow fine-tuning of Hb function in some elasmobranch species (Chong et al., 1999; Naoi et al., 2001).

2.1.1. GENERAL PRINCIPLES OF HEMOGLOBIN STRUCTURE AND FUNCTION

Jawed vertebrate hemoglobins consist of two α -type and two β -type globins that produce a tetramer formed of two $\alpha\beta$ -dimers. Each of the four globin polypeptide chains consists of α -helical segments (named A through H from the N-terminus) that are linked by nonhelical segments (named according to the letters of the flanking helices, i.e., AB through GH), and N- and C-terminal ends are labeled NA and HC, respectively (see Jensen et al., 1998). Between the E and F helices of each chain is the heme pocket that harbors an iron atom bearing heme group that reversibly binds one O₂ molecule; thus, one Hb tetramer can bind up to four O₂ molecules. Although the tetrameric structure of jawed vertebrate Hbs is conserved, the amino acid sequence and length of the polypeptide chains vary among species and vertebrate classes. For example, human HbA, which is the most extensively studied protein, has 141 amino acids in the α -chain and 146 amino acids in the β -chain, whereas the α -chain of elasmobranch Hbs ranges from 140 to 148 residues, and the β -chain, which lacks the D-helix portion that is present in the Hbs of other jawed vertebrates, ranges from 136 to 142 residues (Fig. 3.1). Fig. 3.1 shows the sequences of the major Hb components from eight species of elasmobranchs aligned to human HbA. Hereafter, amino acid residues in elasmobranch Hb will be referenced according to their equivalent numerical position in the α - and β -chains of HbA as shown in Fig. 3.1 (e.g., His F8 β = His 92 β in HbA).

The oxygen equilibrium curve characterizes the relationship between Hb-O₂ saturation and blood PO₂, and the shape and position of the curve reflect Hb-O₂ binding affinity as well as cooperative homotropic interactions among the heme-binding sites. The tetrameric Hbs of vertebrates may assume two distinct conformations: a low-affinity tense (T-state) conformation that occurs in the tissues and a high-affinity relaxed (R-state) conformation that predominates in the lungs or gills (Monod et al., 1965; Perutz, 1970). The binding and release of allosteric effectors (e.g., H⁺, Cl⁻, and organic phosphates) to nonheme sites stabilizes one form over the other, functionally altering Hb-O₂ binding affinity between the sites of O₂ uptake

(A) α -chain



(B) β -chain

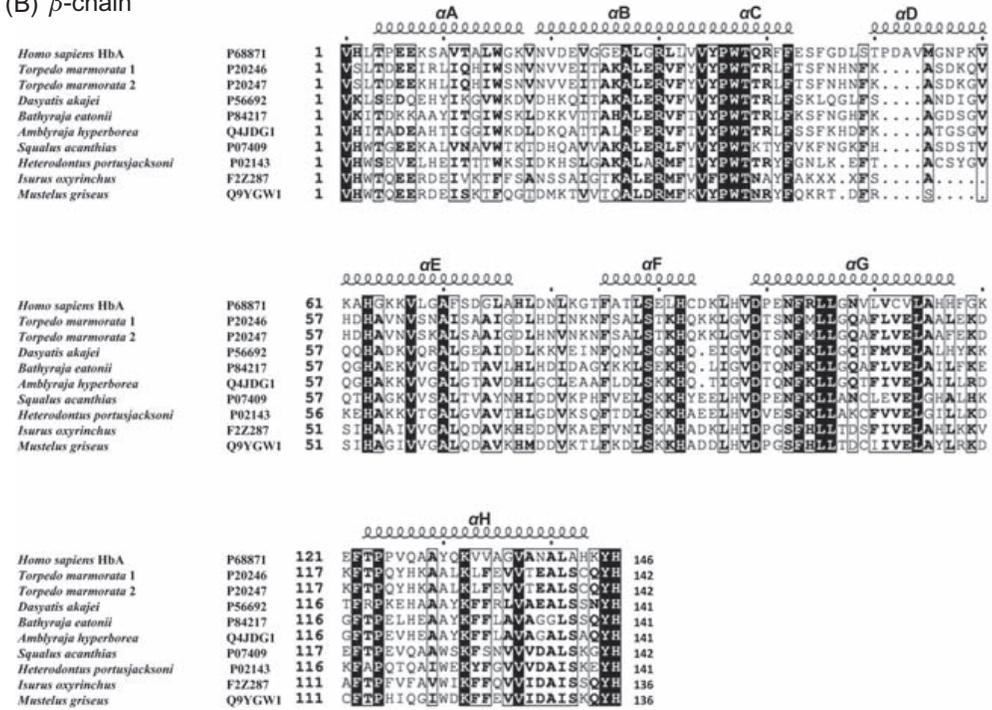


Figure 3.1 (Continued)

and release. The T→R transition is fundamental to the cooperative nature of Hb-O₂ binding, and underlies the sigmoidal shape of the oxygen equilibrium curve because Hb-O₂ affinity increases as successive O₂ molecules are bound. Cooperativity is expressed as the Hill-coefficient (n_H), with values of n_H around unity (~ 1) indicating noncooperative, hyperbolic oxygen equilibrium curves, whereas $n_H > 1$ represents increasing degrees of cooperativity and more sigmoidal curves. Values of n_H for elasmobranch Hb range between 1 and 2 for most species, but reach values as high as 3 (Tables 3.2 and 3.3). A commonly used measure of Hb-O₂ affinity is P_{50} , which is the blood PO_2 that corresponds to 50% Hb-O₂ saturation. The evolution of allosteric modulation of Hb-O₂ affinity through heterotropic interactions with endogenous cofactors (e.g., H⁺, Cl⁻, and organic phosphates) that bind to nonheme sites permits *in vivo* “fine-tuning” of P_{50} between the sites of O₂ loading and offloading.

2.1.2. HEMOGLOBIN MULTIPLICITY

Elasmobranch blood, like that of many bony fishes, contains multiple Hb components (Hb isoforms or isoHbs), ranging in number from 2 to as many as 13 isoforms in hemolysates from some species (Manwell, 1963; Andersen et al., 1973; Bonaventura et al., 1974a; Nash and Thompson, 1974; Fyhn and Sullivan, 1975; Martin et al., 1979; Dickinson and Gibson, 1981; Weber et al., 1983a; Dafré and Reischl, 1990; Galderisi et al., 1996; Dafré and Reischl, 1997; Larsen et al., 2003; Verde et al., 2005). Intraspecific phenotypic variation (Hb polymorphism) is also apparent among hemolysates (Bonaventura et al., 1974a; Fyhn and Sullivan, 1975; Martin et al., 1979; Galderisi et al., 1996), however, the functional significance, if any, is not clear. There does not appear to be substantial functional “division of labor” within elasmobranch hemolysates, because all Hb isoforms are functionally similar (Andersen et al., 1973; Pennelly et al., 1975), or Hb isoforms that differ from the bulk of the hemolysate account for only a small portion of the hemolysate (Dickinson and Gibson, 1981). For example, the

Figure 3.1. Sequence alignment of hemoglobin from eight elasmobranchs aligned to human HbA. The spirals and letters above the sequences identify the α -helices, and black dots correspond to every tenth amino acid residue of HbA. The amino acid residues in black boxes are identical in all sequences, and the residues in white boxes are similar in all sequences. Hemoglobin sequences were retrieved from the UniProt database, aligned using Clustal Omega, and edited for print using ESPript 3 (Gouet et al., 2003). UniProtKB accession numbers for both the (A) α - and (B) β -chains of each species Hb are listed after the species name. Ac indicates that the α -amino group of the α -chain is acetylated (i.e., *Mustelus griseus* and *Heterodontus portusjacksoni*). For *Amblyraja hyperborea* the first three residues of α -chain and first residue of the β -chain were filled in according to Verde et al. (2005).

Table 3.2
Hemoglobin characteristics of elasmobranchs

| Species | Sample | °C | pH | P_{50} | n_H | Φ ($\frac{\Delta \log P_{50}}{\Delta pH}$) | Buffer/Notes | References |
|---|--|------|-----|-----------|-------|--|---|----------------------|
| Batoidea | | | | | | | | |
| Myliobatiformes | | | | | | | | |
| <i>Dasyatis akajei</i> | Stripped isoHb (dominant component) | 24 | 6.5 | 27.9 | 1.9 | -0.41 | 0.06 M heme, 0.05 M Tris/ bis-Tris, 0.1 M Cl ⁻ | Chong et al. (1999) |
| | | 24 | 7.4 | 11.8 | 2.1 | | | |
| | | 24 | 8.5 | 4.2 | 2.1 | | | |
| | +2 mM ATP | 24 | 6.5 | 61.4 | 1.3 | -0.58 ^a | | |
| | +2 mM ATP | 24 | 7.4 | 18.7 | 2.1 | | | |
| | +2 mM ATP | 24 | 8.5 | 4.3 | 2.0 | | | |
| <i>Dasyatis americana</i> | Hemolysate | 25.5 | 7.4 | 15.0 | 1.7 | | 0.033 M phosphate buffer | McCutcheon (1947) |
| <i>Dasyatis centroura</i> | Hemolysate | 25.5 | 7.4 | 15.0 | 1.6 | | 0.033 M phosphate buffer | McCutcheon (1947) |
| <i>Dasyatis sabina</i> | Hemolysate | 25.5 | 7.4 | 15.3 | 1.6 | | 0.033 M phosphate buffer | McCutcheon (1947) |
| <i>Dasyatis say</i> | Hemolysate | 25.5 | 7.4 | 13.5–14.5 | 1.5 | | 0.033 M phosphate buffer | McCutcheon (1947) |
| <i>Gymnura micrura</i> (= <i>Pteroplatea micrura</i>) | Hemolysate | 25.5 | 7.4 | 13.0–14.5 | 1.6 | | 0.033 M phosphate buffer | McCutcheon (1947) |
| <i>Potamotrygon sp.</i> | Stripped hemolysate | 20 | 7.6 | 1 | 1–1.6 | -0.4 | 0.06 mM heme, 0.05 M Tris, Φ and n_H from pH 6.0–9.0 | Martin et al. (1979) |
| | +1 mM ATP | 20 | 7.5 | 3.4 | | | | |
| <i>Rhinoptera bonasus</i> (= <i>R. quadriloba</i>) | Hemolysate | 25.5 | 7.4 | 14.0 | 1.2 | | 0.033 M phosphate buffer, pH 7.4 | McCutcheon(1947) |

| | | | | | | | | |
|--|---------------------|----|-----|-------|-------|---|--|----------------------------|
| Rajiformes | | | | | | | | |
| <i>Amblyraja hyperborea</i> (= <i>Raja hyperborea</i>) | IsoHb I | 2 | 7.5 | 12.9 | | 0 | 0.5–1.0 mM heme, 0.1 M Hepes, 0.1 M NaCl | Verde et al. (2005) |
| | | 10 | 7.5 | 24.4 | | | | |
| | +3 mM ATP | 2 | 7.5 | 13.2 | | | | |
| | +3 mM ATP | 10 | 7.5 | 19.9 | | | | |
| <i>Bathyraja eatonii</i> | IsoHb I | 2 | 7.5 | 14.5 | | 0 | 0.5–1.0 mM heme, 0.1 M Hepes, 0.1 M NaCl | Verde et al. (2005) |
| | | 10 | 7.5 | 28.9 | | | | |
| | +3 mM ATP | 2 | 7.5 | 13.1 | | 0 | | |
| | +3 mM ATP | 10 | 7.5 | 33.5 | | | | |
| <i>Raja eglanteria</i> | Hemolysate | 25 | 6.8 | 37–45 | | | 0.05 M phosphate buffer | Hall and McCutcheon (1938) |
| | | 25 | 7.1 | 38 | | | | |
| | | 25 | 7.4 | 26 | | | | |
| Torpediniformes | | | | | | | | |
| <i>Torpedo nobiliana</i> | Stripped hemolysate | 20 | 7.5 | 15.8 | 1–1.5 | 0 | 0.9 mM heme, 0.05 M Tris. bis-Tris | Bonaventura et al. (1974a) |
| | +4 M NaCl | 20 | 7.5 | 2.8 | 1–1.5 | | | Bonaventura et al. (1974a) |
| | +1 mM ATP | 20 | 7.5 | 16.6 | 1–1.5 | 0 | | |
| | Stripped hemolysate | 20 | 7.5 | 14.4 | 1.5 | | 0.05 M Tris, 0.1 M NaCl | Bonaventura et al. (1974b) |
| | +5 M urea | 20 | 7.5 | 9.6 | 1.4 | | | |
| | + 4M NaCl | 20 | 7.5 | 2.9 | | | | |
| Selachimorpha | | | | | | | | |
| Carcharhiniformes | | | | | | | | |
| <i>Carcharhinus leucas</i> | Stripped hemolysate | 25 | 7.4 | 11 | | | 3% Hb, potassium phosphate buffer | Burke (1974) |
| | | 25 | 6.8 | 17 | | | | |

(Continued)

Table 3.2 (Continued)

| Species | Sample | °C | pH | P_{50} | n_H | Φ ($\frac{\Delta \log P_{50}}{\Delta pH}$) | Buffer/Notes | References |
|---|-----------------------------|----|-----|----------|-------|--|---|----------------------------|
| <i>Carcharhinus plumbeus</i> | Hemolysate | 26 | 7.5 | 5.6 | | | 0.1 M Tris, no difference between unstripped and stripped hemolysates | Brill et al. (2008) |
| | +133 mM urea | 26 | 7.5 | 5.7 | | | | |
| | +5.0 mM ATP | 26 | 7.5 | 26.5 | | | | |
| | +5.0 mM ATP and 133 mM urea | 26 | 7.5 | 26.2 | | | | |
| <i>Galeorhinus galeus</i> (= <i>G. australis</i>) | Stripped hemolysate | | 6.7 | 10.9 | | | 0.031 mM Hb, 0.05 M bis-Tris, 0.3 M NaCl, 0.36 M urea | Coates et al. (1978) |
| | | | 7.3 | 6.9 | | | | |
| | +1.86 mM ATP | | 6.7 | 27.5 | | | | |
| | +1.86 mM ATP | | 7.3 | 19.5 | | | | |
| | +1.86 mM IMP | | 6.7 | 9.8 | | | | |
| <i>Mustelus canis</i> | Hemolysate | 25 | 6.8 | 12–13 | | | 0.05 M Phosphate buffer | Hall and McCutcheon (1938) |
| | | 25 | 7.1 | 9 | | | | |
| | | 25 | 7.4 | 7 | | | | |
| | Stripped hemolysate | 20 | 7.5 | 2.2 | 2.0 | | 0.05 M Tris, 0.1 M NaCl | Bonaventura et al. (1974b) |
| | +2.5 M urea | 20 | 7.5 | 2.1 | 2.0 | | | |
| | | | | | | | | |

| | | | | | | | | | | |
|---|---------------------|---|------|------------------|-------------------------------|---|--|-------------------------------|--------------------------|-------------------|
| <i>Mustelus griseus</i> | Stripped hemolysate | 25 | 6.5 | 8.1 | 2.5 | -0.19 ^a | 0.06 mM heme, 0.05 M Tris/bis-Tris, 0.1 M NaCl | Naoi et al. (2001) | | |
| | | 25 | 7.4 | 5.9 | 2.3 | | | | | |
| | | 25 | 8.5 | 3.4 | 1.8 | | | | | |
| | | +2 mM ATP | 25 | 6.5 | 20.1 | | | | 2.4 | -0.35 |
| | | +2 mM ATP | 25 | 7.4 | 10.6 | | | | 2.6 | |
| <i>Mustelus mustelus</i> | Hemolysate | 25.5 | 7.4 | 7.5 | 1.3 | 0.033 M phosphate buffer | McCutcheon (1947) | | | |
| | | <i>Negaprion brevirostris</i> (= <i>Hypoprion brevirostris</i>) | 25.5 | 7.4 | 7.6 | | | 1.5 | 0.033 M phosphate buffer | McCutcheon (1947) |
| <i>Sphyrna tiburo</i> | Hemolysate | 25.5 | 7.4 | 7.0 | 1.2 | 0.033 M phosphate buffer, pH 7.4 | McCutcheon (1947) | | | |
| <i>Triakis scyllium</i> (= <i>T. scyllia</i>) | Stripped hemolysate | 20 | 6.8 | 5.9 ^a | 1.1 | 0.1 M Bis-Tris HCl | Kono and Hashimoto (1977) | | | |
| | | +ATP | 20 | 6.8 | 7.1 ^a | | | 1.2 | | |
| | | +GTP | 20 | 6.8 | 9.7 ^a | | | 1.1 | | |
| Hexanchiformes | | | | | | | | | | |
| <i>Notorynchus cepedianus</i> | Stripped hemolysate | 6.7 | 10.4 | | | 0.03 mM Hb, 0.05 M bisTris-HCl, 0.3 M NaCl, 0.36 M urea | Coates et al. (1978) | | | |
| | | | 7.3 | 8.2 | | | | | | |
| | | +1.86 mM ATP | 6.7 | 12.1 | | | | | | |
| | | +1.86 mM ATP | 7.3 | 10.9 | | | | | | |
| | | +1.86 mM IMP | 6.7 | 10.0 | | | | | | |
| | +1.86 mM IMP | 7.3 | 8.9 | | | | | | | |
| Lamniformes | | | | | | | | | | |
| <i>Lamna nasus</i> | IsoHb V | 10 | 1.5 | 1.9 | | 0.20–0.33 mM heme, 0.1 M Hepes buffer | Larsen et al. (2003) | | | |
| | | 26 | 2.5 | 1.2 | | | | | | |
| | | +ATP | 10 | 9.7 | 2.3 | | | [ATP]/[Hb _a] > 30 | | |
| | +ATP | 26 | 7.4 | 2.1 | [ATP]/[Hb _a] > 30 | | | | | |

(Continued)

Table 3.2 (Continued)

| Species | Sample | °C | pH | P_{50} | n_H | Φ ($\frac{\Delta \log P_{50}}{\Delta \text{pH}}$) | Buffer/Notes | References |
|--------------------------|--------------------------------------|----|------|----------|-------|---|---|----------------------|
| | IsoHb III | 10 | | 0.9 | 1.8 | + 0.5 -0.6 | Φ from pH 7.5 to 8.3 Φ pH < 7.5 | |
| | +ATP | 26 | | 2.2 | 1.2 | 0 | | |
| | +ATP | 10 | | 9.9 | 2.4 | -0.76 | Φ from pH 7.0 to 7.3, [ATP]/[Hb ₄] > 30 | |
| | +ATP | 26 | | 8.4 | 2.1 | -0.3 | Φ from pH 7.0 to 7.3, [ATP]/[Hb ₄] > 30 | |
| Squaliformes | | | | | | | | |
| <i>Squalus acanthias</i> | Stripped hemolysate | 10 | 7.85 | 2.3 | 1.1 | -0.21 | 0.3–0.4 mM heme, 0.05 M Tris/bis-Tris | Weber et al. (1983a) |
| <i>Squalus suckleyi</i> | Purified hemolysate (crystalline) | 20 | 6.7 | 28 | 1.0 | -0.34 | Potassium phosphate buffer | Manwell (1963) |

P_{50} refers to the PO_2 (mmHg) at which hemoglobin is 50% saturated with O_2 , n_H refers to the Hill coefficient at 50% hemoglobin saturation, and Φ refers to the Bohr coefficient ($\Delta \log P_{50} / \Delta \text{pH}$). °C and pH refer to the conditions under which P_{50} was determined. The “Sample” column describes the type of hemoglobin solution and any added cofactors. “Buffers/Notes” refers to the buffers included in the hemoglobin solution and conditions under which n_H , and Φ were made.

^aIndicates respective parameter was estimated from data or figure in reference.

Table 3.3
Whole-blood characteristics of chondrichthyan fishes.

| Species | P_{aO_2} | P_{vO_2} | P_{aCO_2} | P_{vCO_2} | [Hb] (g dl ⁻¹) | Hct (%) | MCHC (g l ⁻¹) | P_{50} | n_H | Φ | pHa | pHv | Comments | References |
|-------------------------------|------------|------------|-------------|-------------|-------------------------------|---------|------------------------------|----------|------------------|--------|------|-----|------------------------------------|----------------------------|
| HOLOCEPHALI | | | | | | | | | | | | | | |
| Chimaeriformes | | | | | | | | | | | | | | |
| <i>Chimaera monstrosa</i> | | | | | 2.7 | 15.7 | 170 | | | | | | | Larsson et al. (1976) |
| <i>Hydrolagus collicii</i> | | | | | 2.9–3.4 | | | 16 | 1.1 ^a | Absent | | | P_{CO_2} 2.5–28 mmHg, 11°C | Hanson (1967) |
| ELASMOBRANCHII | | | | | | | | | | | | | | |
| Batoidea | | | | | | | | | | | | | | |
| Myliobatiformes | | | | | | | | | | | | | | |
| <i>Dasyatis guttata</i> | | | | | 8.2 | 21.7 | 389 | | | | | | | Filho et al. (1992b) |
| <i>Dasyatis sabina</i> | 90 | 14.2 | | | 14.6 | | | | | | | | 23°C, cannulated | Cameron et al. (1971) |
| | | | | | 3.6 | 24.3 | | | | | | | | Dabruzzi and Bennett, 2014 |
| <i>Dasyatis say</i> | | | | | 3.6 | 14.3 | 235 | | | | | | | Filho et al. (1992b) |
| <i>Myliobatis californica</i> | 87.4 | | 0.6 | | 5.8 | 19.3 | 301 | | | | 7.93 | | 11°C, cannulated, resting | Hopkins and Cech (1994a) |
| | | | | | 5.4 | 23 | | 6.0 | 0.8 | -0.45 | | | pH 8.37, P_{CO_2} 0.2 mmHg, 8°C | |
| | | | | | 5.4 | 23 | | 12.8 | 1.1 | -0.45 | | | pH 7.63, P_{CO_2} 7.6 mmHg, 8°C | |
| | | | | | 5.8 | 23 | | 7.5 | 0.7 | -0.47 | | | pH 8.33, P_{CO_2} 0.2 mmHg, 14°C | |
| | | | | | 5.8 | 23 | | 17.3 | 1.1 | -0.47 | | | pH 7.55, P_{CO_2} 7.6 mmHg, 14°C | |
| | | | | | 5.5 | 23 | | 13.5 | 1.1 | -0.52 | | | pH 7.92, P_{CO_2} 0.2 mmHg, 20°C | |
| | | | | | 5.5 | 23 | | 24 | 1.3 | -0.52 | | | pH 7.45, P_{CO_2} 7.6 mmHg, 20°C | |
| | | | | | 5.2 | 20 | | 12 | 1.1 | -0.47 | | | pH 7.99, P_{CO_2} 0.2 mmHg, 26°C | |
| | | | | | 5.2 | 20 | | 20.3 | 1.3 | -0.47 | | | pH 7.51, P_{CO_2} 7.6 mmHg, 26°C | |

(Continued)

Table 3.3 (Continued)

| Species | PaO ₂ | PvO ₂ | PaCO ₂ | PvCO ₂ | [Hb] (g dl ⁻¹) | Hct (%) | MCHC (g l ⁻¹) | P ₅₀ | n _H | Φ | pHa | pHv | Comments | References |
|---|------------------|------------------|-------------------|-------------------|-------------------------------|---------|------------------------------|-----------------|----------------|---------|-------|-----|---|-----------------------------|
| <i>Myliobatis goodie</i> | | | | | 4.9 | 18.0 | 298 | | | | | | | Filho et al. (1992b) |
| <i>Potamotrygon motoro</i> | | | | | 3.1 | 13.5 | 230 | 4.6 | 1.2–1.4 | –0.05 | | | Washed RBCs, pH 7.4, Φ calculated from pH 6.5–8.0, 29°C | Johansen et al. (1978) |
| <i>Potamotrygon motoro</i> (= <i>P. circularis</i>) | | | | | 5.2 | 23.0 | 226 | 6.7 | 1.3 | –0.26 | | | Washed RBCs, pH 7.4, Φ calculated from pH 6.5–8.0, 29°C | Johansen et al. (1978) |
| (= <i>P. laticeps</i>) | | | | | 3.8 | 8.0 | 475 | 8.1 | 1.3–1.7 | –0.25 | | | Washed RBCs, pH 7.4, Φ calculated from pH 6.5–8.0, 29°C | Johansen et al. (1978) |
| <i>Potamotrygon sp.</i> | | | | | | 24 | | 12.3 | | Present | | | No CO ₂ , pH 7.7, 30°C | Martin et al. (1979) |
| | | | | | | | | 17.0 | | | | | P _{CO₂} 42.6 mmHg, 30°C | Martin et al. (1979) |
| <i>Potamotrygon spp.</i> | | | | | | | | 11.8–12.7 | | –0.19– | –0.26 | | No CO ₂ , 30°C | Powers et al. (1979b) |
| <i>Rhinoptera bonasus</i> | | | | | | | | 16.6–19.7 | | | | | P _{CO₂} 43 mmHg, 30°C | |
| | | | | | | | | | | –0.41 | | | Washed RBCs, Φ calculated from pH 6.9–8.0, 20°C | Scholnick and Mangum (1991) |
| <i>Rhinoptera bonasus</i> | | | | | 6.7 | 25.1 | 286 | | | | | | | Filho et al. (1992b) |
| <i>Taeniura lymna</i> | | | | | 4.8 | 14.8 | 318 | | | | | | | Baldwin and Wells (1990) |
| <i>Trygonoptera testacea</i> | | | | | 3.7 | 21 | 173 | | | | | | 19°C | Cooper and Morris (1998) |
| Rajiformes | | | | | | | | | | | | | | |
| <i>Amblyraja radiata</i> (= <i>Raja radiata</i>) | | | | | 3.8 | 16.7 | 216 | | | | | | | Larsson et al. (1976) |

| | | | | | | | | | | | | | | | | | | |
|--|-----|----|-----|------------------|------|------------------|-----------------|------|-------|-------|----------|------|--|---|--|--|--|---------------------------------|
| <i>Atlantoraja castelnaui</i> (= <i>Raja castelnaui</i>) | | | | 2.6 | 15.4 | 205 | | | | | | | | | | | | Filho et al. (1992b) |
| <i>Atlantoraja cyclophora</i> (= <i>Raja cyclophora</i>) | | | | 5.9 | 17.9 | 366 | | | | | | | | | | | | Filho et al. (1992b) |
| <i>Atlantoraja platana</i> (= <i>Raja platana</i>) | | | | 4.1 | 16.3 | 234 | | | | | | | | | | | | Filho et al. (1992a) |
| <i>Aptychotrema rostrata</i> | 82 | | 1.9 | 3.0 ^b | 12.2 | 242 ^b | 47.6 | 2.1 | | | 7.8 | | | 28°C, P_{50} <i>in vivo</i> , cannulated, respirometer | | | | Speers-Roesch et al. (2012a) |
| <i>Dipturus batis</i> (= <i>Raja batis</i>) | | | | 2.9 | 19.0 | 154 | | | | | | | | | | | | Larsson et al. (1976) |
| <i>Leucoraja naevus</i> (= <i>Raja naevus</i>) | | | | 2.4 | 23.0 | 106 | | | | | | | | | | | | Leray (1982) |
| <i>Leucoraja ocellata</i> (= <i>Raja ocellata</i>) | 70 | 14 | 1.3 | 2.6 | | 20.0 | | | | | 7.82 | 7.67 | | | | | | Dill et al. (1932) |
| | | | | | | | 11 ^a | | | | | | | P_{CO_2} 1 mmHg, 0.2°C | | | | |
| | | | | | | | 20 ^a | 2.0 | | | | | | P_{CO_2} 1 mmHg, 10°C | | | | |
| | | | | | | | 45 ^a | | | | | | | P_{CO_2} 1 mmHg, 25°C | | | | |
| | | | | | | | 95 ^a | | | | | | | P_{CO_2} 1 mmHg, 38°C | | | | Dill et al. (1932) |
| <i>Leucoraja ocellata</i> (= <i>Raja ocellata</i>) | 100 | | 0.8 | | 2.8 | 12.5 | 239 | 27.6 | 1.8 | -0.29 | 7.83 pHi | | | pH 7.82, P_{CO_2} 0.75 mmHg, 12°C, cannulated, flow- through chamber | | | | Graham et al. (1990) |
| | | | | | | | | 34.6 | 2.0 | -0.29 | 7.4 | | | pH 7.40, P_{CO_2} 7.50 mmHg, 12°C | | | | |
| <i>Glaucostegus typus</i> (= <i>Rhinobatos batillum</i>) | | | | 3.8 | 10.5 | 373 | | | | | | | | | | | | Baldwin and Wells (1990) |
| | | | | 3.9 | 13.8 | 281 | 14.8 | 2.2 | -0.08 | | | | | Washed RBCs, pH 7.8, no CO ₂ , 25°C | | | | Wells et al. (1992) |
| | | | | | | | 16.1 | 1.7 | | | | | | Washed RBCs, pH 7.4, no CO ₂ , 25°C | | | | |
| <i>Glaucostegus typus</i> (= <i>Rhinobatos typus</i>) | | | | 3.2 | 14.4 | 220 | | | | | | | | 22-24°C | | | | Lowe et al. (1995) |

(Continued)

Table 3.3 (Continued)

| Species | PaO ₂ | PvO ₂ | PaCO ₂ | PvCO ₂ | [Hb] (g dl ⁻¹) | Hct (%) | MCHC (g l ⁻¹) | P ₅₀ | n _H | Φ | pHa | pHv | Comments | References |
|---|------------------|------------------|-------------------|-------------------|-------------------------------|---------|------------------------------|-----------------|----------------|-------|------|-----|--|--------------------------|
| <i>Raja clavata</i> (= <i>Raja clavata</i>) | 58 | | | | | | | 30.2 | 2.5 | -0.25 | 7.7 | | pH 7.7, 15°C, cannulated | Hughes and Wood (1974) |
| <i>Raja microocellata</i> | | | | | 2.9 | 21.1 | 113 | | | | | | | Leray (1982) |
| <i>Rajella lintea</i> (= <i>Raja lintea</i>) | | | | | 3.4 | 19.3 | 172 | | | | | | | Larsson et al. (1976) |
| <i>Rhinobatos horkelii</i> | | | | | 6.1 | 18.9 | 260 | | | | | | | Filho et al. (1992b) |
| <i>Rhinobatos percellens</i> | | | | | 4.4 | 13.3 | 329 | | | | | | | Filho et al. (1992b) |
| <i>Rioraja agassizii</i> (= <i>Raja agassizii</i>) | | | | | 4.5 | 17.7 | 231 | | | | | | | Filho et al. (1992b) |
| <i>Sympterygia acuta</i> | | | | | 4.7 | 21.6 | 226 | | | | | | | Filho et al. (1992b) |
| <i>Sympterygia bonapartii</i> (= <i>S. bonapartei</i>) | | | | | 3.2 | 13.8 | 241 | | | | | | | Filho et al. (1992b) |
| Torpediniformes | | | | | | | | | | | | | | |
| <i>Narcine brasiliensis</i> | | | | | 4.0 | 17.1 | 227 | | | | | | | Filho et al. (1992b) |
| <i>Torpedo marmorata</i> | 70 | | | | | | | 20.2 | | -0.32 | 7.82 | | pH 7.8, 15°C, cannulated, respirometer 20°C | Hughes (1978) |
| <i>Zapteryx brevirostris</i> | | | | | 4.9 | 19.0 | 247 | 28 ^a | | | | | | Filho et al. (1992b) |
| Selachimorpha | | | | | | | | | | | | | | |
| Carcharhiniformes | | | | | | | | | | | | | | |
| <i>Carcharhinus brevipinna</i> (= <i>C. maculipinnis</i>) | | | | | 7.2 | 30.1 | 265 | | | | | | | Filho et al. (1992b) |
| <i>Carcharhinus limbatus</i> | | | | | 8.4 | 22.3 | 278 | | | | | | | Filho et al. (1992b) |
| <i>Carcharhinus melanopterus</i> | | | | | 4.14 | 17.1 | 243 | | | | | | | Baldwin and Wells (1990) |
| | | | | | 4.11 | 17.0 | 242.9 | 11.1 | 1.7 | -0.35 | | | Washed RBCs, pH 7.8, no CO ₂ , 25°C | Wells et al. (1992) |
| | | | | | | | | 17.9 | 2.2 | -0.35 | | | pH 7.4 | |

| | | | | | | | | | | | | | | | | | | |
|---|---------------|-----|------------------|------|------------------|------|-----|-------|--|-----------|------|--|--|--|--|--|--|--|
| <i>Carcharhinus obscurus</i> | | | 6.2 | 18.2 | 345 | | | | | | | | | | | | | Emery (1986) |
| | | | 4.8 | 15.0 | 324 | | | | | | | | | | | | | Filho et al. (1992b) |
| <i>Carcharhinus plumbeus</i> | | | 5.1 | 14.9 | 350 | | | | | | | | | | | | | Emery (1986) |
| <i>Carcharhinus plumbeus</i> (= <i>C. milberti</i>) | | | 4.4 | 16.1 | 311 | | | | | | | | | | | | | Filho et al. (1992b) |
| | | | 4.01 | 17.7 | 228 | 20.3 | 2.4 | -0.56 | | | | | | | | | | Brill et al. (2008) |
| | | | 4.40 | 21.4 | 206 | | 2.4 | -0.37 | | | | | | | | | | Exercise stressed, pH 7.64, P_{CO_2} 1.5 mmHg, 25°C |
| <i>Carcharhinus porosus</i> | | | 5.8 | 29.9 | 248 | | | | | | | | | | | | | Filho et al. (1992b) |
| <i>Cephaloscyllium</i> <i>isabellum</i> (= <i>C. isabella</i>) | | | 3.5 ^b | 16.8 | 209 ^b | 4.83 | | -0.49 | | | | | | | | | | Filho et al. (1992b) Tetens and Wells (1984) |
| <i>Cephaloscyllium</i> <i>ventriosum</i> | 0.5 – 14.5 | | 3.0 ^b | 15.0 | 208 ^b | 8.3 | 1.5 | -0.32 | | | | | | | | | | Filho et al. (1992b) |
| <i>Galeocerdo cuvier</i> | | | 2.7 | 13.5 | | | | | | 7.60–8.04 | | | | | | | | King (1995) |
| | | | 6.5 | 19.8 | 338 | | | | | | | | | | | | | Emery (1986) |
| | | | | | | | | -0.38 | | | | | | | | | | Scholnick and Mangum (1991) |
| | | | | | | | | | | | | | | | | | | Washed RBCs, Φ calculated from ~pH 6.9–7.9, 20°C |
| <i>Mustelus fasciatus</i> | | | 4.9 | 23.5 | 222 | | | | | | | | | | | | | Filho et al. (1992b) |
| <i>Mustelus schmitti</i> | | | 4.2 | 20.4 | 221 | | | | | | | | | | | | | Filho et al. (1992b) |
| <i>Negaprion acutidens</i> | | | 5.5 | 18.2 | 300 | | | | | | | | | | | | | Baldwin and Wells (1990) |
| | | | 3.6 | 13.0 | 277 | 9.9 | 1.7 | -0.24 | | | | | | | | | | Washed RBCs, pH 7.8, no CO ₂ , 25°C |
| | | | | | | | | | | | | | | | | | | Washed RBCs, pH 7.4, no CO ₂ , 25°C |
| <i>Negaprion brevirostris</i> | 32.5 | 7.1 | 3.6 | 14.9 | | 11.8 | | -0.36 | | 7.72 | 7.54 | | | | | | | pH 7.62, P_{CO_2} 0 mmHg, 24°C, 15% Tris buffer, cannulated, rest/ free swimming |
| | | | | | | | | | | | | | | | | | | Bushnell et al. (1982) |
| <i>Prionace glauca</i> | | | 5.0 | 15.2 | 332 | | | | | | | | | | | | | Emery (1986) |
| | | | 2.9 | 11 | 264 | | | | | | | | | | | | | Exercise stressed Wells et al. (1986) |

(Continued)

Table 3.3 (Continued)

| Species | P_{aO_2} | P_{vO_2} | P_{aCO_2} | P_{vCO_2} | [Hb] (g dl ⁻¹) | Hct (%) | MCHC (g l ⁻¹) | P_{50} | n_H | Φ | pHa | pHv | Comments | References |
|---------------------------------------|------------|------------|-------------|-------------|-------------------------------|---------|------------------------------|--------------|-------|--------|--------|------|--|--------------------------|
| <i>Prionace glauca</i> (continued) | 105.4 | 28.1 | | | | 8 | | | | | 7.66 | 7.50 | 20–22°C, cannulated, swimming 0.45 BL/s ⁻¹ , N=1 | Lai et al. (1997) |
| <i>Scyliorhinus canicula</i> | | | | | 5.62 | 21.8 | | 21.5 25.9 | 1.7 | -0.43 | | | pH 7.58, P_{CO_2} 2.2 mmHg, 17°C pH 7.38, P_{CO_2} 7.3 mmHg, 17°C | Pleschka et al. (1970) |
| <i>Scyliorhinus canicula</i> | 90.9 | 21.3 | | | | | | | | | 7.88 | 7.83 | 7°C, P_{iO_2} 140 mmHg, cannulated, restrained | Butler and Taylor (1975) |
| | 114.4 | 34.5 | | | | | | | | | 7.81 | 7.77 | 12°C, P_{iO_2} 140 mmHg | |
| | 97.6 | 32.9 | | | | | | | | | 7.74 | 7.68 | 17°C, P_{iO_2} 131 mmHg | |
| | 13.8 | 6.6 | | | | | | | | | 7.91 | 7.84 | 7°C, P_{iO_2} 43 mmHg | |
| | 16.5 | 7.0 | | | | | | | | | 7.74 | 7.66 | 12°C, P_{iO_2} 42 mmHg | |
| | 16.8 | 6.0 | | | | | | | | | 7.68 | 7.64 | 17°C, P_{iO_2} 39 mmHg | |
| | 95 | 23 | | | | | | | | | 7.76 | 7.71 | 15°C, P_{iO_2} 148 mmHg, cannulated, respirometer | Short et al. (1979) |
| | 31 | 11 | | | | | | | | | 7.68 | 7.59 | P_{iO_2} 77 mmHg | |
| | 91.2 | | 0.55 | | | | | | | | 7.84 | | 15°C, spinalectomized, cannulated | Truchot et al. (1980) |
| | 92.9 | | 1.3 | | 7.3 | 12 | 616 | | | | 7.59 | | 21°C, cannulated, respirometer | Duthie and Tort (1985) |
| | 98.1 | | 1.0 | | 4.8 | 16.9 | 295 | | | | 7.78 | | 15°C, cannulated, flow-through chambers | Wood et al. (1994) |
| | 93 | | 1.1 | | 4 ^b | 16.7 | | | | | 7.78 | | 15°C, cannulated, flow-through chambers | Perry et al. (1996) |
| | | | | | | | | | | | pHi7.3 | | | |
| | | | | | | | | | | | pHi7.3 | | | |

| | | | | | | | | | | | | | | |
|---|------|------|---------|---------|-------------------|-----------|------------------|------|---------|-------|------|---|--|-----------------------------|
| <i>Scyliorhinus stellaris</i> | 81 | 11 | 1.9 | 3.3 | | 16 | | 12 | | 7.76 | 7.66 | pH 7.79, P_{CO_2} 1.4 mmHg, 17°C, anaesthetized, cannulated | Piiper and Schumann (1967) | |
| | 49 | 10 | 2.0 | 2.6 | | | | 16 | 1.8 | 7.78 | 7.71 | 16°C, cannulated, free-swimming P_{CO_2} 1.5 mmHg, 17°C, <i>in vivo</i> and <i>in vitro</i> | Baumgarten-Schumann and Piiper (1968) Piiper and Baumgarten-Schumann (1968) | |
| | 64 | | 2.1 | | | | | | | | | 17.8–19.2°C, cannulated, resting | Piiper et al. (1977) | |
| <i>Sphyrna lewini</i> | | | | | 10.0 | 26.5 | 370 | | | | | | | Emery (1986) |
| | | | | | 8.4 | 27.3 | 343 | | | | | | | Filho et al. (1992b) |
| <i>Sphyrna tiburo</i> | | | | | | 19.9–22.2 | | | | | | 28°C | | Carlson and Parsons (2003) |
| <i>Sphyrna zygaena</i> | | | | | 6.6 | 25.4 | 281 | | | | | | | Filho et al. (1992b) |
| <i>Triakis semifasciata</i> | 66 | 12 | 1.8 | 2.55 | | 18.3 | | 15.3 | 1.1–1.5 | 7.78 | 7.75 | P_{50} and n_{H_1} <i>in vivo</i> at 15°C, blood gases and pH at 19–22°C, cannulated | Lai et al. (1990) | |
| Heterodontiformes | | | | | | | | | | | | | | |
| <i>Heterodontus portusjacksoni</i> | 82 | 22.5 | 2.6–3.2 | 3.5–4.1 | 4.4 | 20 | | 19 | 0 | | | P_{CO_2} 0–1 mmHg, 20°C | Grigg (1974) | |
| | 97.5 | 24.8 | 2.4 | 1.9 | 3.7 | 20 | 188 | | | 7.82 | 7.82 | 19°C, caudal puncture | Cooper and Morris (1998a,b) | |
| | 105 | 33.8 | 2.1 | 2.3 | | 19 | | 14.4 | 2.0 | –0.11 | 7.82 | 7.76 | 19°C, cannulated | Cooper and Morris (2004a,b) |
| Lamniformes | | | | | | | | | | | | | | |
| <i>Alopias vulpinus</i> | | | | | 13.6 | 37.4 | 360 | | | | | | | Emery (1986) |
| | | | | | 11.9 | 33.0 | 360 | | | | | | | Filho et al. (1992b) |
| <i>Carcharias taurus</i> (= <i>Odontaspis taurus</i>) | | | | | 5.9 | 21.9 | 283 | | | | | | | Filho et al. (1992b) |
| <i>Carcharodon carcharias</i> | | | | | 13.5 | 36.0 | 379 | | | | | | | Emery (1986) |
| <i>Isurus oxyrinchus</i> | | | | | 12.1 ^b | 32.4 | 383 ^b | 10.6 | ≈ 1.5 | +0.16 | | | Exercise stressed, pH 7.6, no CO ₂ , 25°C | Wells and Davie (1985) |
| | | | | | 14.3 | 40.8 | 369 | | | | | | | Emery (1986) |

(Continued)

Table 3.3 (Continued)

| Species | PaO ₂ | PvO ₂ | PaCO ₂ | PvCO ₂ | [Hb] (g dl ⁻¹) | Hct (%) | MCHC (g l ⁻¹) | P ₅₀ | n _H | Φ | pHa | pHv | Comments | References |
|---|------------------|------------------|-------------------|-------------------|-------------------------------|-------------------|------------------------------|-----------------|----------------|-------|------|------|---|------------------------------|
| | | | | | 10.5 | 34 | 318 | | | | | | Exercise stressed | Wells et al. (1986) |
| | | | | | 14.0 | 28.7 | 359 | | | | | | | Filho et al. (1992b) |
| | 82.5 | 30.4 | | | | 24 | | | | | 7.44 | 7.29 | 20–22°C, cannulated, swimming | Lai et al. (1997) |
| Orectolobiformes | | | | | | | | | | | | | | |
| <i>Chiloscyllium punctatum</i> | | | | | 6.3 ^c | 20.9 ^c | 278 | | | | | | 24°C | Chapman and Renshaw (2009) |
| | | | | | 7.5 ^d | 27.3 ^d | | | | | | | | |
| <i>Hemiscyllium ocellatum</i> | | | | | 3.64 | 13.4 | 272 | | | | | | | Baldwin and Wells (1990) |
| <i>Hemiscyllium ocellatum</i> (= <i>H. ocellatum</i>) | | | | | 5.59 | 19.7 | 286.0 | 10.7 | 1.9 | -0.29 | | | Washed RBCs, pH 7.8, no CO ₂ , 25°C | Wells et al. (1992) |
| | | | | | | | | 14.2 | 2.2 | -0.29 | | | Washed RBCs, pH 7.4, no CO ₂ , 25°C | |
| | | | | | 5.3 ^c | 19.0 ^c | 274 | | | | | | 24°C | Chapman and Renshaw (2009) |
| | 97.5 | | 1.35 | | 6.2 ^d | 22.8 ^d | | | | | | | | |
| | | | | | 2.9 ^b | 13.4 | 219 ^b | 32.0 | 1.3 | | 7.87 | | 28°C, P ₅₀ <i>in vivo</i> , cannulated, respirometer | Speers-Roesch et al. (2012a) |
| Squaliformes | | | | | | | | | | | | | | |
| <i>Etmopterus spinax</i> | | | | | 3.0 | 18.9 | 168 | | | | | | | Larsson et al. (1976) |
| <i>Somniosus microcephalus</i> | | | | | 3.2 | 20.5 | 156 | | | | | | | Larsson et al. (1976) |
| <i>Squalus acanthias</i> | | | | | 2.9 | 15.3 | 188 | | | | | | | Larsson et al. (1976) |
| | 111 | | 1.7 | | 3.3 | 20.9 | 158 | | | | 7.85 | | 14–15°C, cannulated, resting | Leray (1982) |
| | | | | | 2.7 ^b | 11.6 | 235 ^b | 13.2 | 1.7 | -0.28 | | | pH 7.85, P _{CO₂} 2.2 mmHg, 15°C | Swenson and Maren (1987) |
| | | | | | | | | | | | | | | Wells and Weber (1983) |

| | | | | | | | | | | | | | | | | | | |
|---------------------------|-------|-----|------|-----|---------|-------|-----|----|------------------|--------|------|------|--|--|--|--|-----------------------------|----------------------|
| <i>Squalus cubensis</i> | | | | | 7.3 | 31.0 | 234 | | | | | | | | | | Filho et al. (1992b) | |
| <i>Squalus suckleyi</i> | 68.8 | 8.1 | 2.3 | 3.4 | 2.6–3.4 | 13–26 | | 17 | 1.2 ^a | Absent | 7.47 | 7.36 | pH 7.6, P_{CO_2} 0.5 mmHg, 11°C, cannulated, free-swimming | | | | Lenfant and Johansen (1966) | |
| | 77 | 13 | | | | | | | | | | | 9–10°C, cannulated, restrained | | | | Hanson and Johansen (1970) | |
| | 104 | 14 | | | | | | | | | | | 9°C, cannulated, restrained | | | | Cameron et al. (1971) | |
| (= <i>S. acanthias</i>) | | | | | 3.0 | 14.8 | 208 | | | | | | 12°C | | | | Perry and Gilmour (1996) | |
| (= <i>S. acanthias</i>) | 102 | | 1.3 | | | | | | | | 7.8 | | 11°C, cannulated, flow-through chamber | | | | Richards et al. (2003) | |
| (= <i>S. acanthias</i>) | 117.4 | 6.3 | 1.24 | 1.6 | | | | | | | 7.87 | 7.81 | 13°C, cannulated, flow-through chamber | | | | Gilmour and Perry (2004) | |
| Squantiniformes | | | | | | | | | | | | | | | | | | |
| <i>Squatina argentina</i> | | | | | 4.3 | 23.1 | 246 | | | | | | | | | | | Filho et al. (1992b) |

P_{aO_2} , P_{vO_2} , P_{aCO_2} , and P_{vCO_2} refer to the *in vivo* partial pressure (mmHg) of O_2 and CO_2 in arterial and venous blood, respectively, P_{50} refers to the PO_2 (mmHg) at which hemoglobin is 50% saturated with O_2 , n_H refers to the Hill coefficient at 50% hemoglobin saturation, Φ refers to the Bohr coefficient ($\Delta \log P_{50} / \Delta pH_e$), and pH_a and pH_v refer to the *in vivo* arterial and venous pH, respectively. Comments refer to the conditions under which P_{50} , n_H , and Φ were made, and/or the conditions under which *in vivo* measurements were made.

^aIndicates respective parameter was estimated from data or figure in reference.

^bConverted using a constant for human HbA: $1 \text{ g dL}^{-1} = 0.1551 \text{ mmol Hb}_4 \text{ L}^{-1}$

^cCaptive sharks

^dWild sharks

hemolysate of the salmon shark, *Lamna ditropis* (= *Lamna ditropus*), eluted into four distinct fractions, one of which accounted for only 5% of the total heme but was functionally distinct from the two predominant and functionally similar fractions that comprised 80% of the total heme (Dickinson and Gibson, 1981). Some elasmobranch hemolysates are comprised of electrophoretically anodal Hbs that have “normal” sensitivities to pH, phosphates, and temperature (e.g., Andersen et al., 1973; Fyhn and Sullivan, 1975; Weber et al., 1983a), which is similar to class I teleost, except that the anodal Hbs of teleosts tend to express a marked pH sensitivity (see Jensen et al., 1998, for a classification of teleost Hbs). For example, electrophoresis revealed that the six Hbs that comprise the hemolysate of the spiny dogfish separated into three distinct anodal bands (fractions I+II, III+IV, and V+VI) with isoelectric points (at 10°C) near pH values of 7.7, 7.4, and 6.9, respectively (Weber et al., 1983a). The fraction containing Hbs III+IV comprised the largest proportion of the hemolysate and exhibited an O₂ affinity similar to the whole hemolysate, but lower than the two less predominant fractions. The main component (III+IV) of *Squalus acanthias* hemolysate also displayed higher heterotropic interactions (i.e., sensitivity to pH and ATP) than the other two components (I+II, and V+VI), and a higher pH sensitivity (i.e., Bohr effect; see Section 2.1.4) than the intact hemolysate (Weber et al., 1983a). However, this situation is unlike the marked heterogeneity typical of class II teleosts that possess a labor force of both cathodal and anodal Hbs with very different sensitivities to pH, phosphates, and temperature (Jensen et al., 1998; Fago et al., 2002; Brauner and Val, 2005). Blood from the regionally heterothermic porbeagle shark, *Lamna nasus*, contains seven distinct Hbs, three of which (Hbs V, IV, and III) account for most of the hemolysate, and are functionally similar with isoelectric points (at 16°C) of 7.58, 7.62, and 7.68, respectively (Larsen et al., 2003). In the presence of ATP the Hbs of *Lamna nasus* exhibit a reverse temperature dependency, whereby increasing temperature increased O₂ affinity (see Section 2.1.7), which is similar to class III teleosts (e.g., Atlantic bluefin tuna, *Thunnus thynnus*; Rossi-Fanelli and Antonini, 1960), although class III teleost Hbs display higher pH sensitivities than the ATP dependent pH sensitivity of *Lamna nasus* Hbs (Larsen et al., 2003).

It is likely that the functional heterogeneity observed in some elasmobranch hemolysates results from different reactivity rates between the α - and β -chains (Andersen et al., 1973; Bonaventura et al., 1974a; Brittain et al., 1982) and from Hb multiplicity that may arise from the formation of tetramers that contain more than two types of globins (Galderisi et al., 1996). These Hb hybrids have been proposed as one possible explanation for the high number of Hb isoforms in the hemolysate of the marbled electric ray, *Torpedo marmorata* (Galderisi et al., 1996). *In vitro*, *Squalus acanthias* Hbs exist in equilibrium between oxygenated dimeric and deoxygenated tetrameric Hb

(Fyhn and Sullivan, 1975), and although the Hbs of other elasmobranchs appear to exist as tetramers in both the oxy and deoxy states, an equilibrium between tetrameric and dimeric Hb may contribute to the formation of Hb hybrids, and thus Hb multiplicity (Galderisi et al., 1996). The functional significance of Hb multiplicity, if any exists, has not been thoroughly investigated in the elasmobranchs.

2.1.3. ONTOGENETIC CHANGES TO HEMOGLOBIN

Distinct fetal Hb isoforms that have higher intrinsic O₂ affinities than adult Hbs are present in a number of egg-laying and live-bearing elasmobranchs (Manwell, 1958; Manwell, 1963; Pennelly et al., 1975; Scholnick and Mangum, 1991; King, 1994). The ancestral reproductive mode in elasmobranchs was likely egg-laying (oviparity), but live-bearing (viviparity) and different forms of maternal input including placental viviparity have evolved independently in a number of lineages (Dulvy and Reynolds, 1997; Awruch, 2015). Juvenile (2 week old) *Squalus acanthias* also have higher Hb-O₂ affinities than adult sharks, and fetal isoHbs persist for at least 10 days posthatch in *Cephaloscyllium ventriosum*, which may ensure adequate O₂ extraction from environmental water until Hb concentration and hematocrit (Hct; the percentage of RBCs in blood) increase to adult levels (Weber et al., 1983a; Wells and Weber, 1983; King, 1994). A high Hb-O₂ affinity (see Section 2.3) should benefit pre-hatch and embryonic individuals by enhancing O₂ extraction in the egg case microenvironment or in fetal circulatory systems (Manwell, 1958; Pennelly et al., 1975; King, 1994).

2.1.4. pH AND THE EVOLUTION OF THE BOHR EFFECT

A decrease in blood pH lowers Hb-O₂ affinity (increases P_{50}) in many vertebrates, permitting a relatively rapid rightward shift of the oxygen equilibrium curve associated with CO₂ production during blood capillary transit. This pH dependency of Hb-O₂ affinity is known as the Bohr effect, named for one of its co-discoverers (Bohr et al., 1904). The alkaline Bohr effect refers to a decreased Hb-O₂ affinity that accompanies declining pH, typically between pH values of 9 and 6, whereas the acid or reverse Bohr effect refers to an increase in Hb-O₂ affinity with declining pH at values typically outside the physiological range (below pH \approx 6); the latter is present in some elasmobranch Hbs (e.g., Larsen et al., 2003; Verde et al., 2005). Various aspects of the Bohr effect have been well reviewed (e.g., Riggs, 1988; Giardina et al., 2004; Jensen, 2004; Berenbrink, 2006), so here discussion will be limited to an evolutionary comparison of the magnitude and mechanism of the Bohr effect in elasmobranch Hbs.

The magnitude of the Bohr effect is quantified as either the Bohr coefficient (Φ) or the Haldane coefficient (ΔzH^+), the latter of which describes the number of Bohr protons that are bound per mole of O₂

released from Hb upon deoxygenation at constant pH. If the shape of the oxygen equilibrium curve is symmetrical, and if other allosteric effectors that differentially bind to the T- and R-state Hb conformations are absent, then the Bohr and Haldane coefficients are thermodynamically equivalent (Wyman, 1964) as is shown in the following relationship:

$$\Phi = \frac{\Delta \log P_{50}}{\Delta \text{pH}} = \frac{1}{4} \Delta z \text{H}^+ \quad (3.1)$$

where Φ values are equal to one quarter $\Delta z \text{H}^+$ values that are determined by acid–base titrations of Hb (e.g., Jensen, 1989; Berenbrink et al., 2005; Regan and Brauner, 2010a). According to Eq. (3.1), if $\Delta z \text{H}^+$ is high then a greater number of H^+ ions will be bound upon a shift from the R- to T-state (oxy to deoxy Hb), which corresponds to a larger change in $\log P_{50}$ per unit change in pH (rightward shift of the oxygen equilibrium curve). The magnitude of the Bohr–Haldane effect is likely the product of a species’ physiological demands and its evolutionary history, in that large Φ values may be optimal for acid–base homeostasis and lower values for blood- O_2 transport (Lapennas, 1983; Brauner and Randall, 1998; Berenbrink, 2006). Furthermore, the magnitude of the Bohr effect is additionally influenced by Cl^- , organic phosphates, CO_2 , temperature, and the experimental pH range (e.g., Weber et al., 1983a), all of which have species-specific influences on Hb- O_2 affinity.

The molecular mechanism of the Bohr effect reflects deoxygenation-linked proton binding at several amino acid residues that stabilise the T-state conformation of the α - and β -chains of jawed vertebrate Hbs. In human HbA, the amino acid residues attributed to the alkaline Bohr effect include Val 1 α (NA1), His 122 α (H5), His 2 β (NA2), Lys 82 β (EF6), His 143 β (H21), and His 146 β (HC3) (Perutz, 1983; Berenbrink, 2006; Mairbäurl and Weber, 2012). Because the $\text{p}K_a$ values of many histidine imidazole groups are within physiological pH values (pH 6 to 8) it is likely that the majority of Bohr protons bind to histidine side chains, which is thought to account for about 90% of the alkaline Bohr effect in human HbA measured in the presence of 0.1 M Cl^- (Lukin and Ho, 2004; Berenbrink, 2006). In the T-state conformation, the C-terminal histidine of HbA (His 146 β) accounts for over 60% of the alkaline Bohr effect (in the presence of 0.1 M Cl^-) owing to a salt bridge that forms with Asp94 β in the same subunit (see Berenbrink, 2006). This salt bridge does not form in any of the sequenced elasmobranch Hbs (see below). In some elasmobranch Hbs the Bohr effect persists over a wide pH range (Mumm et al., 1978; Pennelly et al., 1975; Martin et al., 1979; Weber et al., 1983a), and because elasmobranch Hbs have high specific buffer values (Table 3.4) that correlate with an increased number of physiological buffer groups (i.e., titratable histidine residues) (Jensen, 1989;

Table 3.4
Buffer values

| Species | β Whole blood | β Separated plasma | β Hb | ΔzH^+ | References |
|----------------------------------|---------------------|--------------------------|----------------------------|---------------|--|
| Teleostei | | | | | |
| <i>Ameiurus punctatus</i> | -14.3 (25) | -5.8 | | | Cameron and Kormanik (1982) Szebedinszky and Gilmour (2002) |
| <i>A. nebulosus</i> | -15.5 (24) | -5.7 | -0.12 mmol/g Hb/pH unit | | |
| <i>Anguilla rostrata</i> | -10.1 (20) | -2.7 | | | Hyde et al. (1987) |
| <i>Catostomus commersoni</i> | -8.8 (28) | -2.3 | | | Wilkes et al. (1981) |
| <i>Hippoglossoides elassodon</i> | -6.6 (14) | -2.1 | | | Turner et al. (1983) |
| <i>Katsuwonus pelamis</i> | -8.0 (25) | -3.1 | | 4.1 | Tufts and Perry (1998) and Jensen (2001) |
| <i>Oncorhynchus mykiss</i> | -11.2 (41) | -3.1 | | | Perry et al. (1985) |
| | -9.7 (25) | -2.6 | -6.7 | 3.0 | Tufts and Perry (1998) and Berenbrink et al. (2005) |
| | -10.5 (24) | -2.4 | | | Wood et al. (1982) and Gilmour et al. (2002) |
| <i>Platichthys stellatus</i> | -7.0 (25) | -2.9 | | | Wood et al. (1982) |
| <i>Salvelinus fontinalis</i> | -7.5 (35) | -3.3 | | | Packer and Sunkin (1979) |
| Elasmobranchii | | | | | |
| <i>Leucoraja ocellata</i> | -11.0 (13) | -6.6 | | | Tufts and Perry (1998) and Graham et al. (1990) |
| <i>Raja clavata</i> | -10 | | | | Hughes and Wood (1974) |
| <i>Raja rhina</i> | -6.11 (13.5) | -2.83 | | | Gilmour et al. (2002) |
| <i>Myliobatis californica</i> | -14.3 (20) | | | | Hopkins and Cech (1974a) |
| | to -16.4 (23) | | | | |

(Continued)

Table 3.4 (Continued)

| Species | β Whole blood | β Separated plasma | β Hb | ΔzH^+ | References |
|-------------------------------|---------------------|--------------------------|------------|---------------|--|
| <i>Mustelus asterias</i> | | | -11.5 | 1.1 | Berenbrink et al. (2005) |
| <i>Scyliorhinus stellaris</i> | -8.0 | -10 (true plasma) -4.2 | | | Albers and Pleschka (1967) and Piiper et al. (1972) |
| | -8.8 (18) | -2.6 | -11.4 | 0.3 | Tufts and Perry (1998) and Berenbrink et al. (2005) |
| <i>Squalus acanthias</i> | 10-12 (pH 7.85) | | -11.7 | 1.0 | Weber et al. (1983a) and Berenbrink et al. (2005) |
| <i>Squalus suckleyi</i> | -9.0 (13-26) | -6.5 | | | Tufts and Perry (1998) and Lenfant and Johansen (1966) |
| <i>Triakis semifasciata</i> | -9.3 | | | | Lai et al. (1990) |

β Whole blood and β separated plasma refers to buffer values in Slykes ($\text{mmol HCO}_3^- \text{ pH unit}^{-1} \text{ L}^{-1}$), and are taken from Tufts and Perry (1998) and the listed references. β Hb refers to the hemoglobin buffer value (mol H^+ per mol Hb_4 and pH) in organic phosphate-free, deoxygenated hemolysates at physiological pH and Cl^- , and are from Jensen (2001) and Berenbrink et al. (2005). ΔzH^+ refers to Haldane coefficients (mol H^+ per mol Hb_4) measured by acid-base titrations on stripped hemolysates and are taken from Berenbrink et al. (2005). Unless otherwise stated, values in parenthesis are hematocrit values from Tufts and Perry (1998).

Berenbrink et al., 2005; Berenbrink, 2006), the manifestation of the Bohr effect over a wide pH range may result from a high number of histidine residues that have different pK_a values (Mumm et al., 1978; Aschauer et al., 1985; Weber et al., 1983a). In contrast, teleost fish Hbs generally have low buffer values (Jensen, 1989, 2001; Berenbrink et al., 2005), a characteristic that appears to be strongly associated with a reduction in the number of titratable histidine residues and the evolution of a large Bohr effect and the Root effect (i.e., a decrease in cooperativity and blood oxygen-carrying capacity caused by low pH, even at high PO_2) (Root, 1931; Pelster and Randall, 1998; Berenbrink et al., 2005; Regan and Brauner, 2010a,b). Recent structure–function analyses of species Hb representing all major lineages of jawed vertebrates provide evidence that a low Bohr effect was the ancestral state (i.e., ≤ 1 Bohr proton per Hb₄), and increases in the magnitude of the Bohr effect evolved independently in the amniotes and early actinopterygians, with further increases that evolved in the teleosts, avians, and reptilians due to a reduction in histidine content, and thus specific Hb buffer value (Berenbrink et al., 2005; Berenbrink, 2006). Since the Bohr effect in HbA, and also possibly elasmobranch Hbs, depends on contributions from numerous histidine sites, a reduction in histidine content may result in a decreased Bohr effect (see Berenbrink, 2006). Thus, in HbA the magnitude of the Bohr effect is largely dependent on the salt bridge that forms between His 146 β and Asp94 β in the T-state conformation (see reviews by Kilmartin and Rossi-Bernardi, 1973; Berenbrink, 2006; Mairbäurl and Weber, 2012). In elasmobranchs, the presence of a Bohr effect in many selachian Hbs likely reflects oxygenation-dependent influences from histidine residues, but by different amino acid arrangements than HbA (Aschauer et al., 1985; Chong et al., 1999; Naoi et al., 2001); however, an increase in the magnitude of the Bohr effect caused by a novel mechanism that includes the terminal histidine (His 146 β) appears to have occurred in the myliobatid stingrays (Chong et al., 1999).

Stingrays in the order Myliobatiformes have some of the largest Bohr effects measured in elasmobranchs (Tables 3.2 and 3.3). Of all the sequenced batoidean Hbs, only *Dasyatis akajei* Hb exhibits a marked Bohr effect ($\Phi = -0.41$, and -0.58 in the presence of ATP), which results from amino acid interactions different than that of human HbA (Chong et al., 1999). The salt bridge that forms between His 146 β and Asp 94 β in HbA and contributes to the majority of the Bohr effect cannot form in *Dasyatis akajei* Hb, possibly due a glutamine residue that replaces aspartate at position FG1 in the β -chain (Asp 94 β in HbA) (Chong et al., 1999). Remarkably, pH sensitivity evolved in *Dasyatis akajei* Hb through a hydrogen bond that forms in the T-state between the C-terminal histidine and an asparagine residue at position HC1 β (Lys 144 β in HbA), which is responsible for a large

part of the Bohr effect in this stingray (Chong et al., 1999). Some close relatives of *Dasyatis akajei* also possess Hbs with a Bohr effect (e.g., *Dasyatis sabina*, $\Phi \approx -0.3$ to -0.4 ; Mumm et al., 1978), but the Hbs of other genera of batoideans exhibit little to no Bohr effect (Table 3.2), which indicates that the “stingray Bohr effect” may have evolved within the order Myliobatiformes, possibly representing a further independent evolution of the magnitude of the Bohr effect within jawed vertebrates.

Selachian Hbs exhibit small to moderate Bohr effects (Table 3.2). The small Bohr effects in *Mustelus griseus* Hb ($\Phi = -0.19$) and *Heterodontus portusjacksoni* Hb were attributed to the lack of any considerable interaction between residues that would additionally stabilize the T-state conformation, and acetylation of the free α -amino groups of the α -chain in *Heterodontus portusjacksoni* Hb (Fisher et al., 1977; Nash et al., 1976; Naoi et al., 2001). The slightly larger Bohr effect in *Squalus acanthias* Hb ($\Phi = -0.21$) is consistent with the Haldane coefficient ($\Delta zH^+ = 1.0$) (Weber et al., 1983a; Berenbrink et al., 2005), and likely arises from proton binding to the high number of titratable histidine side chains and the non-acetylated α -chains (Aschauer et al., 1985; Jensen, 1989). The latter is proposed because the conformation of *Squalus acanthias* Hb limits any interaction or bonding between the terminal histidine on the β -chain and other amino acid residues that would additionally stabilise the T-state, similar to *Mustelus griseus* and *Heterodontus portusjacksoni* Hb (Aschauer et al., 1985). Unfortunately, no functional studies were coupled to the structural study of *Isurus oxyrinchus* Hb, but as Aschauer et al. (1985) proposed for *Squalus acanthias* Hb, a nonpolar alanine residue at F6 β (Leu 91 β in HbA) may inhibit salt-bridge formation between AspFG1 β and the terminal histidine, which would prevent any contribution to the overall Bohr effect. This is in line with the observations of Andersen et al. (1973) that Hb-O₂ dissociation rates (carbon monoxide replacement reaction) were independent of pH in *Isurus oxyrinchus* hemolysate. In the isoHbs of the closely related porbeagle shark, *Lamna nasus*, the Bohr effect is also very small or reverse (i.e., acid Bohr effect), but is intensified in the presence of ATP ($\Phi \approx -0.76$ in the presence of ATP). In contrast to these two lamnid sharks, a moderate Bohr effect is present in the stripped hemolysate of the carcharhinid blue shark, *Prionace glauca* ($\Phi \approx -0.4$; Pennelly et al., 1975), but structural studies are lacking for this species' Hbs. Thus, within the elasmobranchs there appears to be unique oxygenation-dependent interactions between histidine residues that contribute to the Bohr effect, and an increase in the magnitude of the Bohr effect by a novel mechanism appears to have evolved in the myliobatid stingrays. Given the paucity of studies conducted to date on the large number of species that exist, there remains a great deal to be learned about the evolution of the Bohr effect in this group.

2.1.5. ORGANIC PHOSPHATE BINDING TO HEMOGLOBIN

The presence of organic phosphates in the RBCs of jawed vertebrates has an additional modulatory effect on the allosteric interaction between O₂ and proton binding sites that increases the magnitude of the Bohr effect of most tetrameric Hbs (Jensen et al., 1998; Val, 2000; Jensen, 2004). The RBCs of fishes are nucleated and thus contain mitochondria that produce the nucleoside triphosphates (NTPs) ATP and GTP, which are the principle allosteric effectors of Hb in most fishes. In contrast, avian RBCs contain inositol pentaphosphate (IPP), and the anucleate RBCs of mammals contains 2,3-bisphosphoglycerate (2,3-BPG). In most elasmobranch RBCs, ATP is the predominant organic phosphate (Leray, 1979; Johansen et al., 1978; Leray, 1982; Weber et al., 1983a; Filho et al., 1992a); however, GTP is the more potent allosteric effector of Hb-O₂ affinity (Kono and Hashimoto, 1977; Weber et al., 1983a), and in some elasmobranch RBCs the concentration of GTP is equal to or greater than that of ATP (Kono and Hashimoto, 1977; Borgese et al., 1978; Bartlett, 1982; Filho et al., 1992a; Wells et al., 1992). Additionally, inosine monophosphate (IMP) has been reported from the RBCs of a number of elasmobranchs, and IPP from the RBCs of *Squalus acanthias* and the electric ray, *Torpedo nobiliana* (Borgese and Nagel, 1978; Coates et al., 1978; Wells et al., 1992). However, IMP does not appreciably decrease Hb-O₂ affinity in either *Notorynchus cepedianus* or *Galeorhinus galeus* (Table 3.2; Coates et al., 1978), and ATP is clearly the predominant NTP in *Squalus acanthias* RBCs (Bartlett, 1982; Weber et al., 1983a). In general, the total NTP concentration and the NTP/Hb ratio are lower in elasmobranchs than teleosts; within the elasmobranchs, selachian RBCs generally contain a greater absolute concentration of NTPs than batoidean RBCs (Filho et al., 1992a). The lower NTP/Hb ratio in elasmobranchs RBCs compared to that of teleosts, and the antagonistic effect of urea on Hb-ATP sensitivity (see below; Weber et al., 1983b), may be the basis for the generally lower whole blood P_{50} values reported for elasmobranchs, but to our knowledge this has not been investigated.

Organic phosphate binding to elasmobranch Hbs reduces Hb-O₂ affinity in most selachians and at least one batoidean species (Table 3.2). In human HbA and most teleost fish Hbs, organic phosphates bind to specific amino acid residues at positions NA1, NA2, EF6, and H21 in the central cavity between the two β -chains, which reduces Hb-O₂ affinity by stabilizing the T-state conformation (Perutz and Brunori, 1982; Gronenborn et al., 1984; Jensen et al., 1998). The amino acid residues in the phosphate binding region of HbA are not conserved in batoidean Hbs, which may explain why most of the skates and rays lack any significant allosteric effect of ATP on Hb-O₂ binding (Bonaventura et al., 1974a; Verde et al., 2005). In contrast, the

β -chains of Hb from the selachians *Squalus acanthias*, *Heterodontus portusjacksoni*, and *Mustelus griseus* possess the same amino acids as human HbA at positions NA1, NA2, and EF6, and a positively charged lysine residue at H21 where HbA has a positively charged histidine (Fig. 3.1). Consequently, the site that binds organic phosphates in HbA and teleost Hbs also has been implicated in binding ATP in the T-state conformation of *Squalus acanthias* and *Mustelus griseus* Hb, which concomitantly decreases Hb-O₂ affinity and increases the magnitude of the Bohr effect for both of these species (Aschauer et al., 1985; Weber et al., 1983a; Naoi et al., 2001). Both ATP and inositol hexaphosphate (IHP) markedly decreased Hb-O₂ affinity in a number of sharks in the orders Carcharhiniformes and Lamniformes (Table 3.2, and see Pennelly et al., 1975), and IHP also reduced Hb-carbon monoxide (CO) affinity in the salmon shark, *Lamna ditropis* (= *Lamna ditrotus*) (Dickinson and Gibson, 1981). It is not clear whether the site of 2, 3-BPG binding in human HbA similarly binds NTPs in the Hbs of lamnid sharks because a serine substitution at H21 β (His 143 β in HbA) of *Isurus oxyrinchus* Hb may inhibit NTP binding in the central cavity between the β chains (Fig. 3.1). However, novel phosphate binding sites may be present in lamnid shark Hbs because in *Lamna nasus* Hbs the oxygenation-dependent release of ATP causes the overall heat that is normally released during Hb-oxygenation to be retained even though heme-oxygenation is intrinsically exothermic (see Section 2.1.7; Larsen et al., 2003).

In at least one stingray, *Dasyatis akajei*, the magnitude of the Bohr effect is increased in the presence of ATP. Remarkably, not only does *Dasyatis akajei* Hb exhibit a novel Bohr effect mechanism, but it also possesses a novel ATP binding site that is located within the central cavity between the two β -chains just inside the 2, 3-BPG binding site of human HbA. In this region, Arg 104 β and Ala 135 β of human HbA are substituted for two positively charged amino acid residues, lysine (G6 β) and arginine (H13 β), respectively, that favor binding of ATP in the T-state conformation (Fig. 3.1; Chong et al., 1999; Verde et al., 2005). Except for *Torpedo marmorata* Hb, all other sequenced elasmobranch Hbs possess positively charged residues at position G6 β (Arg 104 β), but lack a substitution for a positively charged residue at position H13 β (Ala 135 β). Additionally, the presence of ATP does not have a substantial effect on Hb-O₂ affinity for *Torpedo nobiliana*, either of the polar skates, *Amblyraja hyperborea* and *Bathyraja eatonii*, or a freshwater stingray, *Potamotrygon sp.* (Bonaventura et al., 1974a; Martin et al., 1979; Verde et al., 2005). Therefore, the presence of the novel ATP binding site described for *Dasyatis akajei* Hb may have evolved within the family Dasyatidae, although clearly further structure–function studies of Hb from myliobatid rays are required to investigate this hypothesis.

2.1.6. INTERACTIONS OF HEMOGLOBIN WITH UREA AND TMAO

Some elasmobranchs possess urea insensitive globin proteins, a trait that may be crucial for dealing with their high blood urea levels (see Chapter 4; [Ballantyne and Fraser, 2013](#)). This trait, however, is also present in some bony fishes and invertebrate lineages and was thus likely inherited by the elasmobranchs ([Edelstein et al., 1976](#); [Weber et al., 1977](#); [Scholnick and Mangum, 1991](#)). The concentration of urea in the blood plasma of marine elasmobranchs held or captured in seawater ranges from 290 to 490 mM, but RBC intracellular values are higher owing to the fraction of urea bound to Hb ([Browning, 1978](#); [Yancey and Somero, 1980](#); [Tetens and Wells, 1984](#); [Wells et al., 1992](#); [Wood et al., 1994](#); [Brill et al., 2008](#); [Ballantyne and Fraser, 2013](#); see also Chapter 4). Urea concentrations ranging from physiological to pharmacological levels only slightly increased Hb-O₂ affinity or had very little influence on Hb-O₂ affinity for a number of batoideans ([Bonaventura et al., 1974b](#); [Martin et al., 1979](#); [Scholnick and Mangum, 1991](#)) and selachians ([Bonaventura et al., 1974b](#); [Scholnick and Mangum, 1991](#); [Wells et al., 1992](#); [Cooper and Morris, 2004](#); [Brill et al., 2008](#)). However, urea increased Hb-O₂ affinity for the draughtsboard shark, *Cephaloscyllium isabellum* (= *Cephaloscyllium isabella*), and North Sea spiny dogfish, *Squalus acanthias* ([Weber, 1983](#); [Weber et al., 1983a,b](#); [Tetens and Wells, 1984](#)). Curiously, for *Squalus acanthias* captured in the western Atlantic Ocean, Hb-O₂ affinity was almost insensitive to urea ([Scholnick and Mangum, 1991](#)). These reported differences for *Squalus acanthias* Hb may be due to the experimental methods and parameters employed by each group of researchers (i.e., stripped hemolysates used by Weber and colleagues vs. washed and re-suspended RBCs by Scholnick and Mangum), or may reflect real variation that exists among *Squalus acanthias* populations ([Scholnick and Mangum, 1991](#)). The mechanism of Hb-urea binding and the relative sensitivity or insensitivity of elasmobranch Hb to urea has been discussed in some detail and may be at least partially related to the integrity of the tetrameric molecular structure of Hb ([Bonaventura et al., 1974b](#); [Weber, 1983](#); [Weber et al., 1983a,b](#); [Aschauer et al., 1985](#)).

Urea and TMAO differently affect the O₂ affinity and the oxygenation-linked binding of ATP to elasmobranch Hbs. The urea-induced increase in Hb-O₂ affinity of *Squalus acanthias* (North Sea) is more pronounced at pH 7.7 than at pH 7.3, values that are in the range of elasmobranch arterial and venous blood pH values ([Table 3.3](#)), and urea also decreased cooperativity and reduced the ATP sensitivity of *Squalus acanthias* Hb ([Weber et al., 1983a,b](#)). The cooperativity of Hb-O₂ binding was relatively unaffected by urea in other elasmobranch Hbs ([Bonaventura et al., 1974b](#); [Tetens and](#)

Wells, 1984; Scholnick and Mangum, 1991). However, physiologically relevant concentrations of urea also decreased the effect of ATP on Hb-O₂ affinity in *Cephaloscyllium isabellum*, but not in *Carcharhinus plumbeus* (Tetens and Wells, 1984; Brill et al., 2008). While urea influences ligand binding to Hb for some species, TMAO had no discernable effect on the Hb-O₂ equilibria for *Squalus acanthias*, *Heterodontus portusjacksoni*, or *Rhinoptera bonasus* (Weber, 1983; Scholnick and Mangum, 1991; Cooper and Morris, 2004; Kolhatkar et al., 2014). Since urea reduced the O₂ affinity and ATP sensitivity of *Squalus acanthias* Hb (Weber et al., 1983a,b), and urea-TMAO counteraction appears to be absent in *Squalus acanthias* RBCs (Weber, 1983; Kolhatkar et al., 2014), Weber (1983) proposed that ATP subsumes the role of TMAO in counteracting the effects of urea in the RBCs of *Squalus acanthias*. However, urea did not eliminate the effect of ATP on Hb-O₂ affinity in *Squalus acanthias* and *Cephaloscyllium isabellum*. Therefore, Hb-O₂ equilibria studies conducted in the absence of urea may overestimate the allosteric effect that NTPs have on elasmobranchs Hbs (Weber et al., 1983a). Furthermore, since TMAO tends to increase the rigidity of proteins, the lack of a TMAO effect on *Squalus acanthias* Hb function may preserve the conformational changes responsible for the Bohr effect (Weber, 1983), and recent findings indicate that TMAO may play a thermoprotective role in *Squalus acanthias* RBCs (Kolhatkar et al., 2014). Why some selachian species possess urea sensitive Hbs and others do not is unclear. More research is needed to understand this trait and its relevant importance to the urea osmoconforming strategy of elasmobranchs.

2.1.7. ENTHALPY OF HEMOGLOBIN-OXYGENATION

Due to the exothermic nature of heme-oxygenation, rising temperature will usually decrease Hb-O₂ affinity directly. In most fishes, Hb can be considered to carry heat because endothermic deoxygenation in the tissue capillaries and exothermic oxygenation in the gill lamellae result in outward conductive heat transport (Jensen et al., 1998). The temperature sensitivity of Hb-O₂ binding can be quantified by the overall enthalpy (or apparent heat) of oxygenation ($\Delta H'$), which is calculated according to the van't Hoff isochore:

$$\Delta H = 2.303 \cdot R \cdot \frac{\Delta \log P_{50}}{\Delta \frac{1}{T}} \quad (3.2)$$

where R is the gas constant and T is the absolute temperature (Wyman, 1964). Calculation of $\Delta H'$ according to Eq. (3.2) assumes linearity of the van't Hoff plot [$\Delta \log P_{50}/\Delta(1/T)$], but $\Delta H'$ itself can be temperature dependent (Fago et al., 1997; P.R. Morrison, T.S. Harter, R.W. Brill, and C.J. Brauner,

unpublished data on *Carcharhinus plumbeus*). Numerically negative $\Delta H'$ values denote the exothermic release of heat during oxygenation, commonly described as “normal” temperature effects, whereas positive values are indicative of endothermic heat absorption or “reverse” temperature effects. In addition to the intrinsic heat of heme-oxygenation (ΔH^O), the overall enthalpy of oxygenation comprises contributions from the heat of solution of O_2 ($-12.55 \text{ kJ mol}^{-1} O_2$), the heat of conformational changes (transition between the T- and R-states), and heats of ionization and dissociation of allosteric effectors (e.g., protons, phosphates, and Cl^- ions). As is evident in Fig. 3.2 values for ΔH^O (including the heat of solution of O_2) are very similar among the hemolysates of jawed vertebrates (Powers et al., 1979a), which is due to the highly conserved nature of the prosthetic heme groups (i.e., the O_2 binding sites). However, in the presence of allosteric effectors and at low pH where the Bohr effect is operative $\Delta H'$ values clearly differ among species (Fig. 3.2). This adaptive variation in the temperature sensitivity of P_{50} is largely due to variation in oxygenation-linked dissociation of allosteric effectors that have an endothermic contribution to the overall enthalpy of oxygenation, $\Delta H'$ (Weber and Fago, 2004; Weber and Campbell, 2011; Mairbäurl and Weber, 2012).

Oxygenation-linked dissociation of protons (i.e., Bohr protons) contributes endothermically to $\Delta H'$ as is evident in stripped hemolysate from the spiny dogfish, *Squalus acanthias*. The temperature effect (2 and 15°C) decreased between pH 7.9 ($\Delta H' = -44 \text{ kJ mol}^{-1}$) and pH 7.0 ($\Delta H' = -35 \text{ kJ mol}^{-1}$) (Fig. 3.2), which correlates with an increase in the Bohr effect over the same pH range (Weber et al., 1983a). For the predominant Hb component from the polar skates, *Bathyrāja eatonii* and *Amblyrāja hyperborea*, $\Delta H'$ (2 and 10°C) actually becomes more exothermic (i.e., more negative) as pH falls, which reflects oxygenation linked proton binding that is in line with the presence of a reverse (acid) Bohr effect in the Hb of these species (Verde et al., 2005). Above pH 7.5 addition of ATP and NaCl also caused $\Delta H'$ values to become even more exothermic for *Bathyrāja eatonii* Hb, the reverse observed in *Amblyrāja hyperborea* (Verde et al., 2005).

Some regionally heterothermic sharks and teleosts have independently evolved Hbs that bind oxygen with a reduced or reverse temperature sensitivity. Within the regionally heterothermic lamnid sharks, shortfin mako, *Isurus oxyrinchus*, blood P_{50} values exhibit quite a small temperature dependence, but the cold temperate porbeagle shark, *Lamna nasus*, and salmon shark, *Lamna ditropus*, possess Hbs that bind O_2 or CO with a reverse temperature sensitivity (Andersen et al., 1973; Dickinson and Gibson, 1981; Larsen et al., 2003). For two isolated Hb components from the hemolysate of *Lamna nasus*, the enthalpy of oxygenation (10 to 26°C) determined at pH 7.3 and in the absence of allosteric effectors is “normal”

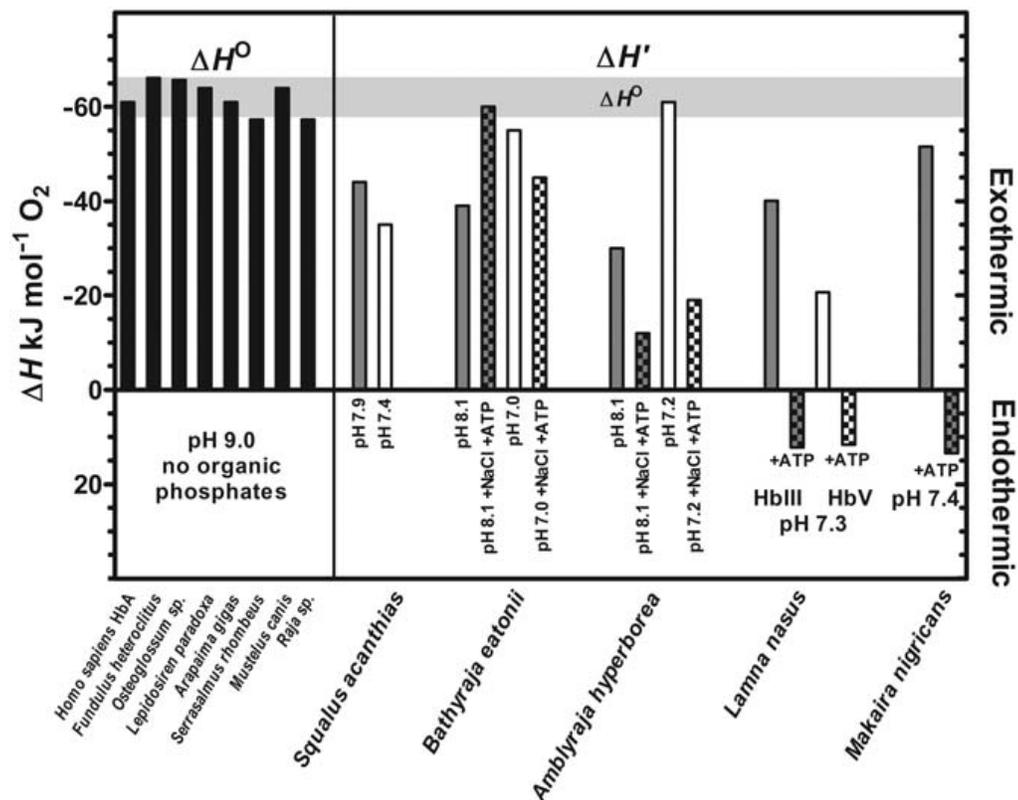


Figure 3.2. Enthalpies of oxygenation (including the heat of solution of O_2 , $-12.55 \text{ kJ mol}^{-1} O_2$) for stripped hemoglobin from representative jawed vertebrates. Black bars to the left of the vertical line are enthalpies of heme-oxygenation (ΔH^O) measured in the absence of organic phosphates and at high pH where the Bohr effect is inoperative (taken from Powers et al., 1979a). To the right of the vertical line are overall enthalpies of oxygenation ($\Delta H'$) for the elasmobranchs *Squalus acanthias* (Weber et al., 1983a), *Bathyraja eatonii*, *Amblyraja hyperborea* (Verde et al., 2005), *Lamna nasus* (Larsen et al., 2003), and a teleost, the blue marlin, *Makaira nigricans* (Weber et al., 2010). Stippling indicates the presence of ATP, and gray and white bars represent high and low pH, respectively, except for *Lamna nasus* where gray and white bars refer to different Hb isoforms. The gray horizontal bar refers to ΔH^O from Powers et al. (1979a), and values of $\Delta H'$ that fall below the gray bar results from species-specific release of allosteric effectors (e.g., protons, Cl^- , and ATP) that contribute endothermically to $\Delta H'$. As indicated on the outer right edge of the figure, negative $\Delta H'$ values are exothermic and positive $\Delta H'$ values are endothermic.

for Hbs III ($\Delta H' = -40 \text{ kJ mol}^{-1}$) and IV ($\Delta H' = -21 \text{ kJ mol}^{-1}$), but reverse in the presence of ATP ($\Delta H' \approx +12 \text{ kJ mol}^{-1}$) (Fig. 3.2; Larsen et al., 2003). The positive increase of $\Delta H'$ is due to endothermic contributions from the release of ATP and protons during transition from the T- to R-state Hb conformations, where the temperature effect is eliminated by ATP in the T-state, but persists at high pH and in the R-state. The bond energies

determined for *Lamna nasus* Hb indicate that ATP may be involved in the formation of an additional salt bridge per heme group that constrains the T-state conformation (Larsen et al., 2003), but determination of the amino acid residues implicated in the reverse temperature effect of lamnid shark Hb awaits elucidation of the Hb structure and sequence in additional species. Regionally heterothermic teleosts, the billfishes (Istiophoridae) and the tunas (Scombridae), also possess Hb(s) with reduced or reverse temperature sensitivities (Rossi-Fanelli and Antonini, 1960; Ikeda-Saito et al., 1983; Weber et al., 2010). The oxygenation linked release of allosteric effectors reduces or eliminates the intrinsic heat of heme-oxygenation (i.e., ΔH°) in the lamnid sharks, tunas, and billfishes. However, variation in the molecular mechanism and allosteric effectors involved indicates that reduced and reverse temperature dependent Hb-oxygenation evolved independently in each of these lineages of fishes (Weber and Campbell, 2011).

2.2. Red Blood Cell Function and Homeostasis

Red blood cells provide the working environment for Hb, and although elasmobranchs tend to have lower hematocrits than teleosts, the RBCs of elasmobranchs are generally 3 to 4 fold larger than those of teleosts, and contain more Hb per RBC (Emery, 1986; Fänge, 1992; Filho et al., 1992b). Consequently, mean corpuscular Hb concentrations (MCHC) are higher in elasmobranchs than in teleosts (Filho et al., 1992b). For all vertebrates, encapsulation of Hb within the RBCs allows control over the Hb microenvironment by creating two functional compartments in the blood: plasma and the RBC intracellular space. The pH and the concentrations of ions, osmolytes, and other molecules can differ significantly between these two compartments. Consequently, electrochemical gradients form between the RBC intracellular and extracellular environments, and these gradients must be actively maintained to prevent RBC volume changes and to control RBC intracellular pH (pHi) to optimize Hb function. The Hb-O₂ affinity within the RBCs is dependent on the intrinsic O₂ affinity of Hb and its sensitivity to heterotropic effectors. By altering RBC, volume, intracellular pHi, and the intracellular concentration of organic phosphates, an organism can “fine-tune” Hb-O₂ affinity in response to changing metabolic and environmental demands.

2.2.1. RED BLOOD CELL ORGANIC PHOSPHATE CONCENTRATIONS

The RBC intracellular concentration of organic phosphates impacts O₂ transport directly, by their allosteric effect on Hb function, and indirectly owing to their influence on RBC pHi (Wood and Johansen, 1973). Organic

phosphate binding to Hb and the concomitant increase in P_{50} is pH dependent for some elasmobranchs (e.g., *Squalus acanthias*, Weber et al., 1983a), so the indirect effects of organic phosphates may be more important at the high plasma pH values in the arterial circulation of elasmobranchs (Table 3.3; Wells and Weber, 1983). Anoxic incubation of *Squalus acanthias* blood caused a small decrease in RBC intracellular ATP concentrations (Bricker et al., 1968; Wells and Weber, 1983), as well as a fall in both P_{50} and plasma pH (Wells and Weber, 1983). Because a decrease in pH would be expected to increase P_{50} , the observed fall may be linked to the decline of ATP. NTP levels were also unchanged in hypercapnic winter skates, *Leucoraja ocellata* (= *Raja ocellata*; Graham et al., 1990), and strenuous exercise failed to provoke a significant hematological response or depletion of ATP and GTP in the RBCs of the giant shovelnose ray, *Glaucostegus typus* (= *Rhinobatos typus*; Lowe et al., 1995). Based on evidence from the few studied species, the intracellular concentrations of NTPs in elasmobranch RBCs are fairly resistant to change, which limits any modulating effect of NTPs on Hb-O₂ affinity and O₂ transport. This contrasts dramatically with that of teleosts (Val, 2000). However, in juvenile sandbar sharks, *Carcharhinus plumbeus*, exhaustive anaerobic exercise was associated with RBC swelling and a reduction of intracellular NTP concentrations, clearly warranting further research on this topic in more species of elasmobranchs (Brill et al., 2008).

2.2.2. RED BLOOD CELL VOLUME AND pH HOMEOSTASIS

Red blood cell volume is primarily determined by the osmotic pressure difference between the intracellular and extracellular environments, which must be actively maintained (see reviews by Nikinmaa, 1990; Nikinmaa and Salama, 1998; Hoffmann et al., 2009). Because the concentration of Na⁺ is higher in plasma than in cells, and RBCs maintain a net negative charge, Na⁺ will “leak” through RBC membranes down its electrochemical gradient. Sodium leak is counteracted, and homeostatic maintenance of RBC volume is maintained, by the sodium pump Na⁺/K⁺-ATPase that pumps Na⁺ out of the cell in exchange for K⁺ (Tosteson and Hoffman, 1960; Bricker et al., 1968; Guerra et al., 1969; Nikinmaa and Salama, 1998). RBC volume responds to changes in blood osmolarity, which is quickly corrected by active control of the solute content of the cell via a regulatory volume decrease (RVD) or a regulatory volume increase (RVI) (Nikinmaa, 1990; Cossins and Gibson, 1997; Hoffmann et al., 2009; Chara et al., 2011). Evidence from experiments on the blood of the little skate, *Leucoraja erinacea*, and spiny dogfish, *Squalus acanthias*, indicates that during hypotonic stress the band 3 anion exchanger functions as an osmolyte channel to extrude taurine as part of the RVD mechanism (Perlman and

Goldstein, 2004). The signal for RVD taurine efflux appears to be decreased intracellular ionic strength that results from a hyposmotic medium (Wittels et al., 2000; Koomoa et al., 2001; Perlman and Goldstein, 2004). Signaling occurs via protein kinase C and inositol phosphate systems, which trigger ion and organic solute transport, including efflux of taurine and ATP from the RBC (McConnell and Goldstein, 1988; Goldstein and Brill, 1991; Goldstein et al., 2003). Extracellular ATP and other nucleotides released from the RBC may then modulate osmolyte release during hyposmotic stress (Goldstein et al., 2003; Perlman and Goldstein, 2004). A hyposmotic extracellular environment also stimulates Na^+ -dependent, and Na^+ -independent uptake of TMAO in *Leucoraja erinacea* RBCs, and an acute thermal stress increased TMAO levels in *Squalus acanthias* RBCs (Wilson et al., 1999; Kolhatkar et al., 2014). Volume-activated efflux of TMAO is triggered as part of the RVD mechanism, and the Na^+ -independent movement of TMAO across the RBC is thought to occur through the same channel as taurine (Wilson et al., 1999; Koomoa et al., 2001). In the model proposed by Perlman and Goldstein (2004) for an RVD in the RBCs of *Leucoraja erinacea*, the reduction in intracellular ionic strength caused by increased cell volume triggers tyrosine kinase phosphorylation of anion exchanger dimers that come together to form a tetrameric osmolyte channel (Puffer et al., 2006). This oligomerization is accompanied by an interaction with cytoplasmic proteins that causes the formation or exposure of a high-affinity ankyrin binding site and the dissociation of band 4.1, which in turn initiates the efflux of taurine (Perlman and Goldstein, 2004; Perlman et al., 2006). While this RVD pathway has been extensively described in *Leucoraja erinacea*, and to a lesser extent in *Squalus acanthias*, whether this process is conserved among elasmobranchs in general remains to be investigated.

Elasmobranch RBCs regulate pH by way of rapid anion exchange (Obaid et al., 1979) and a high Hb buffer value (Jensen, 1989; Graham et al., 1990; Berenbrink et al., 2005). Buffering of H^+ by Hb decreases the charge of Hb, and because elasmobranch (and teleost) RBC membranes are more permeable to Cl^- than to Na^+ or K^+ , a decrease in the net negative charge of impermeable polyions (e.g., Hb and organic phosphates) causes a net influx of Cl^- and osmotically obliged water, effectively alkalizing the intracellular environment and causing cell swelling (Nikinmaa and Salama, 1998). Some ion transporting pathways are also apparently oxygen dependent (Bogdanova et al., 2009); thus, the osmotic state and volume of the RBCs may be partially dependent on the arterial-venous PO_2 difference. For example, RBC swelling occurred in anoxia-exposed epaulette sharks, *Hemiscyllium ocellatum*, but when ambient PO_2 was restored to normoxic levels, RBC volume was restored, indicating that oxygen-dependent mechanisms may be involved in regulation of RBC volume in some species

of elasmobranch, possibly in response to internal hypoxic conditions (Chapman and Renshaw, 2009).

There is no published evidence that elasmobranch RBCs possess secondarily active ion transporters such as β -adrenergic Na^+/H^+ exchangers (β -NHEs) (Tufts and Randall, 1989; Wood et al., 1994; Lowe et al., 1995; Berenbrink et al., 2005; Brill et al., 2008), which are characteristic of teleosts that possess pH-sensitive Root effect Hbs (Motais et al., 1992; Berenbrink et al., 2005). Thus, active RBC swelling beyond steady-state volumes and uncoupling of plasma pH from RBC pH do not appear to occur in elasmobranch RBCs. For example, hypercapnic winter skates, *Leucoraja ocellata* (= *Raja ocellata*) exhibited no signs of RBC pHi regulation or RBC swelling in response to elevated PCO_2 (Graham et al., 1990). The lack of RBC pHi regulation may be partially attributed to the high buffering capacity of elasmobranch Hb, which restricts any large changes in RBC pHi caused by secondarily active H^+ transport, because of the large flux of protons that would be required to cause such a change (Nikinmaa, 1997).

2.3. Blood-Oxygen Content: Hemoglobin Concentration and Hematocrit

Blood O_2 -carrying capacity is directly related to blood Hb concentration and thus Hct, wherein 1 g of Hb can bind 1.35 mL of O_2 (1 mmol L^{-1} of Hb_4 binds 4 mmol L^{-1} of O_2). The influence of Hb concentration on blood- O_2 content over a range of blood PO_2 is illustrated in Fig. 3.3 for six elasmobranchs compared to a mammal (humans) and a high performance swimming teleost, Atlantic bluefin tuna (*Thunnus thynnus*). In elasmobranchs, Hb concentration and Hct generally appear to be lower than in teleosts (Hall and Gray, 1929; Larsson et al., 1976; Filho et al., 1992b; Gallagher and Farrell, 1998); however, elasmobranchs tend to have a higher MCHC (Filho et al., 1992b). In elasmobranchs, RBCs may be released into the circulation from the sites of RBC production (erythropoiesis) such as the spleen, epigonal organ, and Leydig's organ (i.e., the hemopoietic organs) (e.g., Fänge and Mattisson, 1981; Fänge, 1992). During exercise or exposure to environmental stressors (e.g., hypoxia, and low salinity) fishes can increase Hct either by increasing the concentration of circulating RBCs and/or by increasing RBC volume (e.g., see review by Gallagher and Farrell, 1998). In exercising teleosts, Hct is increased by adrenergic stimulation of the spleen to release RBCs, but it is not clear whether the spleen of elasmobranchs functions in this capacity (Opdyke and Opdyke, 1971; Nilsson et al., 1975; Lowe et al., 1995).

The effects of environmental O_2 on Hb concentrations and Hct levels in elasmobranchs has not been thoroughly investigated, but the limited evidence indicates that some elasmobranchs may alter Hct or increase

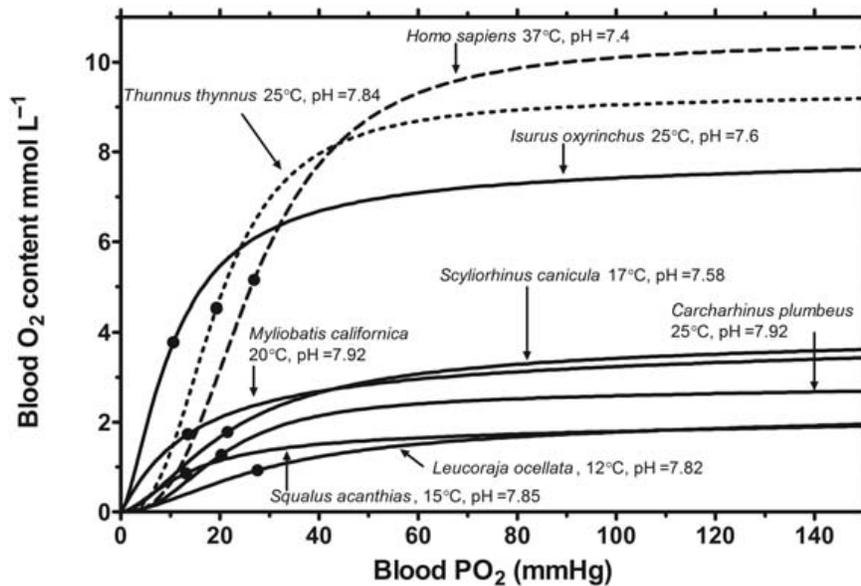


Figure 3.3. Comparison of the relationship between blood- O_2 content (mmol L^{-1}) and blood PO_2 (mmHg) for six elasmobranch species (solid lines), Atlantic bluefin tuna (dotted line; *Thunnus thynnus*), and humans (dashed line; *Homo sapiens*). Blood- O_2 content was determined at corresponding blood PO_2 levels by using Hill's equation, $\alpha b P_b + 4[\text{Hb}_4] \frac{P_b^n}{P_{50}^n + P_b^n}$ (Hill, 1921; but see Malte and Weber, 1985), where αb is the oxygen solubility of blood plasma as a function of temperature (from Boutiler et al., 1984), P_b is the blood PO_2 , $[\text{Hb}_4]$ is the molar concentration of tetrameric Hb (Hb_4), P_{50} is the blood PO_2 at 50% Hb- O_2 saturation, and n is the Hill coefficient. Data for the elasmobranch species are from Table 3.2 (Pleschka et al., 1970; Wells and Weber, 1983; Wells and Davie, 1985; Graham et al., 1990; Hopkins and Cech, 1994a; Brill et al., 2008), *Thunnus thynnus* data are from Brill and Bushnell (2006), and data for *Homo sapiens* from Reeves (1980). Black circles denote species P_{50} values.

blood O_2 -carrying capacity in response to low environmental O_2 . For example, anoxia exposure increased Hct for both the epaulette shark, *Hemiscyllium ocellatum*, and the gray carpet shark, *Chiloscyllium punctatum*, but the cause of this hematological response was different for each species (Chapman and Renshaw, 2009). Whereas the increased Hct for *Hemiscyllium ocellatum* was coupled to a decline in MCHC, which is symptomatic of RBC swelling in response to anoxia, *Chiloscyllium punctatum* increased the concentration of RBCs in circulation, but the relative influences of RBC release versus hemoconcentration were not investigated (Chapman and Renshaw, 2009). In contrast, progressive hypoxia had no effect on Hct, Hb concentration, or MCHC in *Hemiscyllium ocellatum* or the shovelnose ray, *Aptychotrema rostrata* (Speers-Roesch et al., 2012a), and acute hypoxia exposure had no effect on Hct or Hb concentration in Atlantic stingrays, *Dasyatis sabina* (Dabruzzi and Bennett, 2013). Similarly, no change in Hct was observed in hypoxic or hypercapnic *Squalus suckleyi* (= *Squalus*

acanthias; Perry and Gilmour, 1996). The effects of exercise and salinity on Hct are discussed in Sections 2.4.2 and 2.4.4, respectively.

In at least one species of elasmobranch, blood O₂-carrying capacity is higher in developing rather than in post-hatch and adult individuals. In the oviparous swellshark, *Cephaloscyllium ventriosum*, Hct and Hb concentration increased over development (pre-hatch to a few months old), and these changes were associated with an increase in P_{50} (decreased Hb-O₂ affinity) over the same developmental stages (King, 1994). King (1994) speculated that a high blood O₂-carrying capacity would be more beneficial for free-swimming adult sharks than pre-hatch sharks that are restricted to the potentially hypoxic microenvironment within the egg case. We are not aware of any other published studies that tracked Hct, Hb concentration, or oxygen levels within the egg case over the course of development for other elasmobranch species, although Manwell (1958) reported that in big skates, *Raja binoculata*, egg case PO₂ rarely dropped below half that of seawater.

Remarkably, Hb and Hct levels in the regionally heterothermic sharks are comparable to those of the regionally heterothermic tunas, and to mammalian values (Table 3.3 and Fig. 3.3; e.g., Reeves, 1980; Lapennas and Reeves, 1982; Reeves et al., 1982; Bernal et al., 2001), although there is no clear relationship between activity level and these hematological indices among sampled elasmobranch species (Emery, 1986; Baldwin and Wells, 1990; Gallagher and Farrell, 1998). In contrast, a comprehensive study of hematological parameters for 80 fishes, including 28 elasmobranchs, noted an apparent correlation between Hb concentration and the activity level of elasmobranchs (Filho et al., 1992b). Furthermore, a high Hb concentration has been implicated as one of a few modifications to the oxygen cascade that underlies a higher activity level in cownose rays, *Rhinoptera bonasus*, relative to Atlantic stingrays, *Dasyatis sabina* (Grim et al., 2012). However, we advise caution against making such general assumptions, especially without a quantitative gauge of a species' "activity level," or perhaps more applicably the ability of a species to increase its level of activity (e.g., aerobic scope). Additionally, because the relationship between "activity level" and O₂ consumption is complex, vertebrates have evolved a number of different ways to increase O₂ delivery, and apparent modifications to components involved in the oxygen cascade may be related to other physiological or environmental demands, as well as to a species' evolutionary history, and not necessarily strictly related to blood O₂-carrying capacity and O₂ delivery *per se*. Additionally, the heat transporting role of Hb may also influence Hb concentrations and Hct for a number of fishes. The high Hct and Hb concentrations typical of the large, athletic, regionally-heterothermic sharks and teleosts (see reviews by Gallagher and Farrell, 1998; Bernal et al., 2001) may be related to the endothermic

mechanisms of heat production and retention that these species have evolved (Gibson and Carey, 1982; Carey and Gibson, 1983), and not just to their athleticism and large scope for increased aerobic activity.

2.4. Whole-Blood-Oxygen Equilibria

Vertebrate respiratory systems are complex, emergent organizations of biological components, with each component carrying out multiple jobs to maintain whole animal homeostasis. Although *in vitro* studies of hemoglobin solutions are central to interpreting the evolution, physiological significance, and mechanisms involved in allosteric modulation of Hb-O₂ affinity, experiments on whole blood provide insight into *in vivo* function at the level of the whole animal. The *in vivo* Hb P_{50} values of water-breathing fishes often dictate what part of the water column a species can exploit and the type of environment where it can survive. A low P_{50} (high Hb-O₂ affinity) typically is associated with hypoxia-tolerant fishes as it will enhance O₂ loading at the gills and ensure that Hb is maximally saturated at low environmental PO_2 (Mandic et al., 2009; Wells, 2009; Speers-Roesch et al., 2012a). In contrast, a high P_{50} (low Hb-O₂ affinity) is advantageous for offloading O₂ at high PO_2 in the tissue capillary beds, which will increase O₂ diffusion between the blood and a mitochondrion in the tissues, but can compromise O₂ loading at the gills if ambient water PO_2 is low. Measured under physiologically-relevant conditions, the whole blood P_{50} values of elasmobranchs generally range from 10 to 20 mmHg (Table 3.3), similar to or slightly lower than those of most teleosts. Interestingly, the P_{50} of the spotted ratfish, *Hydrolagus colliei*, is also within this range (16 mmHg; Hanson, 1967), while those of the batoideans *Leucoraja ocellata* (27.6 mmHg; Graham et al., 1990) and *Raja clavata* (30.2 mmHg; Hughes and Wood, 1974) are quite high, similar to mammalian values (e.g., human $P_{50} \approx 27$ mmHg; Reeves, 1980). Although elasmobranchs do not generally exhibit the diversity of blood respiratory characteristics that is typical of teleosts, there does appear to be considerable interspecific variation in whole blood P_{50} , and as discussed below, in response to changing internal and environmental conditions.

2.4.1. CARBON DIOXIDE AND BLOOD PH

Metabolic carbon dioxide diffuses into the tissue capillary blood, elevating venous PCO_2 and potentially affecting blood-oxygen transport owing to the concomitant decrease in blood pH. Blood-oxygen transport in many elasmobranchs appears to be relatively insensitive to CO₂ (e.g., Graham et al., 1990), although some species exhibit modest Bohr effects *in vitro* (Table 3.3). The CO₂ Bohr coefficient describes the changes in blood pH

caused by altering PCO_2 , and it is this relationship between Hb- O_2 affinity and CO_2 -dependent changes in whole blood pH (i.e., extracellular or plasma pH; pHe) that is most commonly reported in whole blood- O_2 equilibria studies. The relationship between pHi and pHe is usually linear, but deviates from unity. If so, the extracellular Bohr coefficient (Φ_{pHe}) can be divided by the $\Delta pHi/\Delta pHe$ value to estimate the true or intracellular Bohr coefficient (Φ_{pHi}). Interestingly, in the few species of elasmobranchs that have been investigated, $\Delta pHi/\Delta pHe$ is close to unity, unlike teleosts. The whole blood Bohr coefficient for *Scyliorhinus canicula* ($\Phi_{pHe} = -0.43$; Pleschka et al., 1970) is only slightly increased when the $\Delta pHi/\Delta pHe$ value (~ 0.94 ; Wood et al., 1994) is taken into account ($\Phi_{pHi} = -0.43/0.94 = -0.46$). Similarly, for *Squalus acanthias* blood the relationship between pHi and pHe is slightly nonlinear over the measured pH range but $\Delta pHi/\Delta pHe$ averages about 0.9, which only slightly underestimates the Bohr coefficient ($\Phi_{pHe} = -0.28$ and $\Phi_{pHi} = -0.28/0.9 = -0.31$; Wells and Weber, 1983).

The CO_2 Bohr effect differs from the fixed-acid Bohr effect, which is usually reported in studies of hemolysates or isolated Hb and describes changes in P_{50} caused by titration with non- CO_2 or fixed acid. Inequality between the CO_2 and fixed-acid Bohr coefficients indicates that CO_2 also has a specific effect on Hb- O_2 affinity that is caused by carbamate formation from the preferential binding of CO_2 to the α -amino groups of deoxygenated Hb (Kilmartin and Rossi-Bernardi, 1973; Jensen et al., 1998). As in teleosts, the α -amino groups of the α -chains are acetylated in at least two elasmobranch Hbs (Fig. 3.1), and the α -amino groups of the β -chains are likely involved in organic phosphate binding in some selachian Hbs (see Section 2.1.5). Consequently, oxylabile carbamino formation may affect blood- O_2 transport in some elasmobranchs more than others (Jensen, 2004). In *Squalus acanthias*, the whole blood Bohr effect is slightly greater than the fixed-acid Bohr effect measured on stripped hemolysates (Weber et al., 1983a; Wells and Weber, 1983), which is consistent with the allosteric interaction of RBC intracellular organic phosphates and possibly a specific CO_2 effect because CO_2 can potentially form carbamate with the non-acetylated α -chains of *Squalus acanthias* Hb (Weber et al., 1983a; Wells and Weber, 1983; Aschauer et al., 1985).

Butler and Metcalfe (1988) provided a brief review of published Bohr coefficients for elasmobranchs; here, this information will be updated. Bohr coefficients for stripped Hb and hemolysates are listed in Table 3.2, and those for whole blood are tabulated in Table 3.3. Interestingly, blood from the spotted ratfish, *Hydrolagus colliei*, shows no indication of cooperative O_2 binding ($n_H = 1.1$) and CO_2 tensions as high as 27 mmHg did not affect n_H or P_{50} , indicating the lack of a Bohr effect in the blood of this holocephalan (Hanson, 1967). The Bohr effect also is absent or very small for a number of

elasmobranchs, including the batoideans *Leucoraja ocellata*, *Torpedo nobiliana*, *Amblyraja hyperborea*, and *Bathyraja eatonii*, and the selachians *Mustelus canis*, *Heterodontus portusjacksoni*, and *Squalus suckleyi* (Tables 3.2 and 3.3; Dill et al., 1932; Lenfant and Johansen, 1966; Bonaventura et al., 1974a; Grigg, 1974; Scholnick and Mangum, 1991; Cooper and Morris, 2004; Verde et al., 2005). However, the presence of a fixed-acid Bohr effect in purified Hb from *Squalus suckleyi* (Table 3.2; Manwell, 1963) contradicts the absence of a Bohr shift in whole blood from this shark (Lenfant and Johansen, 1966), and consequently it may be worth revisiting species reported in the older studies.

Whole blood Bohr coefficients (Φ_{pHc}) in elasmobranchs are typically lower than those reported for teleosts, ranging from -0.05 to -0.52 in the batoideans, and from -0.11 to -0.56 in the selachians (Table 3.3). Wells and Davie (1985) reported a slight reverse Bohr effect in blood from mako sharks, *Isurus oxyrinchus*, but the data were probably not representative of resting conditions because the sharks had been so exhaustively exercised that air-equilibrated blood had a pH of 6.2! Interestingly, a Bohr effect is present in whole blood from the marbled electric ray, *Torpedo marmorata* (Hughes, 1978), but is absent from stripped hemolysates of the congeneric *Torpedo nobiliana* (Bonaventura et al., 1974a). Whole blood from the bat eagle ray, *Myliobatis californica*, exhibits quite a substantial Bohr effect (Table 3.3; Hopkins and Cech, 1994a), and because all other rays in the order Myliobatiformes that have been studied to date (i.e., *Dasyatis akajei*, *Dasyatis sabina*, *Potamotrygon* spp., *Rhinoptera bonasus*) also possess a Bohr effect (see Tables 3.2 and 3.3) it seems reasonable to hypothesize that the mechanism responsible for the Bohr effect in *Dasyatis akajei* Hb (see Section 2.1.4) may have been inherited from the common ancestor of the Myliobatid families. The magnitude of the Bohr effect in *Myliobatis californica* is relatively temperature independent, whereas in the draughts-board shark, *Cephaloscyllium isabellum*, the whole blood Bohr coefficient was greater in 5°C acclimated sharks than in 15°C acclimated sharks (Table 3.3; Tetens and Wells, 1984). Some studies have reported P_{50} values and Bohr coefficients measured on washed RBCs that were resuspended in buffered elasmobranch saline (e.g., Scholnick and Mangum, 1991; Wells et al., 1992), but it is not known whether this method compromises the integrity of elasmobranch RBCs (e.g., see Caldwell et al., 2006).

The Bohr effect is generally considered beneficial for tissue O_2 delivery. Lapennas (1983) proposed that a Bohr coefficient equal to half of the respiratory quotient (RQ , = CO_2 eliminated/ O_2 consumed) should be optimal for O_2 delivery to the tissues of the dog and the gray seal. Even though the assumptions (i.e., steady-state conditions, the absence of a specific CO_2 effect on Hb- O_2 affinity, and a RQ between 0.7 and 1.0) of

Lapennas' "optimal" Bohr coefficient hypothesis are rarely met in fishes, it provides a starting point to evaluate the potential benefit of the Bohr effect. In teleosts, tissue O₂ delivery is likely enhanced by the Root effect that reduces blood O₂-carrying capacity at low pH (Rummer et al., 2013; Randall et al., 2014). Elasmobranchs lack a physiologically relevant Root effect (Lenfant and Johansen, 1966; Pennelly et al., 1975; Farmer et al., 1979; Ingermann and Terwilliger, 1982; Wells and Weber, 1983; Wells and Davie, 1985; Dafré and Wilhelm, 1989; Berenbrink et al., 2005; Brill et al., 2008), but some elasmobranchs (e.g., *Myliobatis californica*, *Rhinoptera bonasus*, *Carcharhinus plumbeus*, and *Scyliorhinus canicula*) have whole blood Bohr coefficients (Table 3.3) that are very similar to mammalian values ($\Phi \approx -0.46$ to -0.51 ; Reeves, 1980; Lapennas and Reeves, 1982; Reeves et al., 1982). Thus, it is very likely that the Bohr effect in elasmobranchs was under selection to take advantage of the arterial-venous pH difference (Table 3.3) to enhance O₂ delivery to the tissues.

2.4.2. EXERCISE

Exercise generally increases the metabolic demand of muscle for O₂, requiring an increase in blood O₂ transport if O₂ supply is to match demand. Transport and supply of O₂ can be increased by increasing blood flow (cardiac output, \dot{Q}) or by extracting a greater amount of O₂ from the blood, as described by the Fick principle:

$$\dot{M}O_2 = \dot{Q}(CaO_2 - CvO_2) \quad (3.3)$$

where $\dot{M}O_2$ is total O₂ consumption and $CaO_2 - CvO_2$ refers to the arterial-venous O₂ content difference. Whether blood transport of O₂ can keep up with demand depends on the severity of exercise as well as species-specific Hb characteristics. An exercise-induced decreased pH in the capillary blood will enhance O₂ delivery to the working muscles via the Bohr effect. Increasing the delivery of O₂ through the oxygen cascade can also be achieved by evolutionary or short-term adjustments to any of a number of components of the cardiorespiratory system of fishes (Jones and Randall, 1979). Cardiorespiratory adjustments and the metabolic demand for O₂ in response to exercise have only been investigated in a few species of elasmobranchs (Piiper et al., 1977; Brett and Blackburn, 1978; Bushnell et al., 1982; Lai et al., 1990; Lowe et al., 1995; Richards et al., 2003) primarily due to difficulties associated with working with most species of elasmobranchs in a controlled laboratory environment.

The elasmobranch cardiorespiratory system generally has been regarded as ineffective in matching O₂ supply with demand during acute bouts of exhaustive exercise, owing to a low blood O₂-carrying capacity and the apparent lack of regulated blood-O₂ transport (Piiper et al., 1977; Butler

and Metcalfe, 1988; Lowe et al., 1995; Brill et al., 2008). For example, strenuous exercise (≤ 10 min of burst swimming) did not elicit any changes in Hb concentration, Hct, MCHV, RBC count, RBC volume, or RBC intracellular NTP concentrations in the giant shovelnose ray, *Glaucostegus typus* (= *Rhinobatos typus*), which was suggested to limit post-exercise recovery and the ability of this ray to resume aerobic activity (Lowe et al., 1995). In contrast, Hct increased by 10% in swimming lemon sharks, *Negaprion brevirostris* (5 min of forced swimming at a sustainable pace; Bushnell et al., 1982), and blood from juvenile sandbar sharks, *Carcharhinus plumbeus*, anaerobically exercised on recreational hook-and-line fishing gear exhibited certain “teleost-like” mechanisms that may minimize disruption to blood- O_2 transport caused by metabolic acidosis (Brill et al., 2008). Brill et al. (2008) observed that sandbar sharks suffering from an exercise induced metabolic acidosis as indicated by plasma lactate and pH levels, elevated blood- O_2 carrying capacity via a 21% increase in Hct and a 10% increase in Hb concentration, increased RBC volume by 28%, reduced RBC NTP concentrations by -15% , and were capable of some degree of RBC pHi regulation to maintain Hb- O_2 affinity during a metabolic acidosis (Brill et al., 2008). The assumption that elasmobranchs in general are ineffective at matching O_2 supply with demand during strenuous or exhaustive exercise does not seem to be true of all species. For example, the blood O_2 -carrying capacity of the highly athletic and regionally heterothermic shortfin mako shark, *Isurus oxyrinchus*, far exceeds those measured in other sharks, and makos swimming at a sustainable velocity of 0.45 BL/s^{-1} (body lengths per second) maintain venous O_2 reserves 1.5 times greater than those of leopard sharks, *Triakis semifasciata*, swimming at 0.45 BL/s^{-1} (35% of U_{crit} ; critical or maximal sustained swimming velocity) or resting *Negaprion brevirostris*, although the limits of O_2 transport system have yet to be investigated in *Isurus oxyrinchus* (Bushnell et al., 1982; Lai et al., 1990, 1997). Furthermore, Wells and Davie (1985) observed that blood from severely exercise-stressed *Isurus oxyrinchus* caught during a fishing tournament showed signs of possible RBC swelling following capture, but this observation has not been further investigated.

With the exception of *Negaprion brevirostris* (see below) arterial blood gas levels are largely unaffected during exercise in studied elasmobranchs (Piiper et al., 1977; Lai et al., 1990; Richards et al., 2003). To meet the increased metabolic demands for O_2 that are brought on by swimming, *Triakis semifasciata* and *Scyliorhinus stellaris* draw from their venous O_2 reserves. In *Triakis semifasciata*, arterial Hb- O_2 saturation did not change when sharks transitioned from rest to swimming at 0.45 BL/s^{-1} or 35% of U_{crit} , but venous Hb- O_2 saturation decreased from 39% to 18%, and venous PO_2 (PvO_2) and CvO_2 were reduced by 44% and 56%, respectively

(Lai et al., 1990). Similarly, the O₂ content of mixed venous blood was reduced by 55% in spontaneously swimming *Scyliorhinus stellaris* (Piiper et al., 1977). Unexpectedly, venous O₂ stores were unchanged in swimming *Negaprion brevirostris*, but arterial PO₂ (PaO₂) and CaO₂ increased by 40% and 31%, respectively (Bushnell et al., 1982). This increase of arterial blood-O₂ during exercise in *Negaprion brevirostris* resulted from an increase in Hb saturation from below full at rest to near full saturation during routine swimming (Bushnell et al., 1982), a characteristic that has also been observed in yellowfin tuna, *Thunnus albacares*, an athletic, regionally heterothermic teleost (Korsmeyer et al., 1997). Maintaining low PaO₂ and low arterial Hb-O₂ saturation limits branchial O₂ uptake during rest and routine swimming, but allows O₂ loading over a wider PO₂ range in response to demand (Bushnell et al., 1982; Korsmeyer et al., 1997). This strategy of limiting branchial O₂ exchange may have an osmoregulatory or ionoregulatory benefit in the context of the osmorepiratory compromise because the anatomical characteristics of the gill and its circulation that are favorable for gas exchange are detrimental for osmoregulation (Bushnell et al., 1982; Gonzalez and McDonald, 1992; Korsmeyer et al., 1997).

The apparent lack of a hematological response (i.e., increased Hct or Hb concentration), RBC swelling, or changes to arterial blood gases in some elasmobranchs compared to teleosts may not necessarily limit aerobic activity in elasmobranchs *per se*. The contrasting physiological responses during exercise in elasmobranchs and teleosts may reflect differences in energy and recovery metabolism (Richards et al., 2003; Speers-Roesch and Treberg, 2010; see also Chapter 7), and the evolution of different pathways to deal with the transport and elimination of CO₂ (see below; Tufts and Perry, 1998; Gilmour and Perry, 2010). Additionally, the lack of a Root effect in elasmobranch blood alleviates the requirement to strictly regulate RBC pHi (Tufts and Randall, 1989; Pelster and Randall, 1998). However, clearly the physiological response to exercise is not the same for all elasmobranchs (e.g., Bushnell et al., 1982; Brill et al., 2008), and to our knowledge no studies of blood gas transport and blood respiratory properties for an elasmobranch exercising near maximal aerobic capacity have been published (e.g., see Hillman et al., 2013). This line of research warrants further investigation, particularly in the high-energy-demand, lamnid sharks (Sepulveda et al., 2007; Ezcurra et al., 2012), which display a suite of cardiorespiratory specializations (see Bernal et al., 2001) that very likely can serve to increase O₂ supply upon demand.

2.4.3. HYPOXIA

Hypoxic conditions occur in a wide range of marine and freshwater environments, with the magnitude, cause, and duration of low dissolved O₂

varying among habitat type and location (Diaz and Breitburg, 2009). The level of environmental hypoxia that triggers disruption of physiological function depends upon both the species and physiological system in question (Farrell and Richards, 2009). During exposure to progressive hypoxia, aquatic organisms typically maintain resting $\dot{M}O_2$ as water dissolved O_2 levels decrease to a critical PO_2 (P_{crit}), at which point the organism transitions from oxyregulation to oxyconformation, and $\dot{M}O_2$ then falls with decreasing PO_2 levels. Fishes with greater hypoxia tolerance tend to have lower P_{crit} values than less tolerant fishes (e.g., Mandic et al., 2009; Speers-Roesch et al., 2012a), with hypoxia-tolerance in some elasmobranchs ostensibly being linked to an increased capacity for oxygen uptake and transport at low water PO_2 (Speers-Roesch et al., 2012a). Hemoglobin- O_2 affinity (i.e., P_{50}) is an important determinant of P_{crit} (Mandic et al., 2009), which is strongly associated with O_2 transport during hypoxia exposure in elasmobranchs (Speers-Roesch et al., 2012a). Many species of elasmobranchs have evolved the ability to survive periods of environmental hypoxia, but the particular physiological, anatomical, and behavioral responses to low dissolved oxygen conditions vary among species (see reviews by Butler and Metcalfe, 1988; Perry and Tufts, 1998; Richards et al., 2009). Earlier studies of the responses of elasmobranchs to environmental hypoxia may have been compromised by handling and holding stress, as well as the intrusive instrumentation that was necessary to obtain useful physiological measurements (Ogden, 1945; Satchell, 1961; Hughes and Umezawa, 1968; Piiper et al., 1970; Butler and Taylor, 1971; Butler and Taylor, 1975; Hughes, 1978; Short et al., 1979). In undisturbed and uninstrumented *Scyliorhinus canicula*, hypoxic exposure caused a decrease in swimming activity and an increase in ventilation frequency (Metcalfe and Butler, 1984). A meticulous study on the influence of catecholamine release on the hypoxic response of cannulated *Squalus suckleyi* (= *Squalus acanthias*) found that ventilation amplitude and frequency increased while PaO_2 decreased in response to environmental hypoxia (Perry and Gilmour, 1996). Ventilation frequency of the hypoxia-tolerant epaulette shark *Hemiscyllium ocellatum* also increased during hypoxic exposure (Routley et al., 2002). Decreases in PaO_2 and CaO_2 during exposure to progressive hypoxia followed a similar trend for *Scyliorhinus canicula*, *Squalus suckleyi*, *Hemiscyllium ocellatum*, and *Aptychotrema rostrata* (Butler and Taylor, 1975; Perry and Gilmour, 1996; Speers-Roesch et al., 2012a), indicating that these elasmobranchs have comparable ventilatory responses to hypoxia (Speers-Roesch et al., 2012a).

High water temperature increased $\dot{M}O_2$ and influenced P_{crit} in *Scyliorhinus canicula* (Butler and Taylor, 1975). At 7°C, *Scyliorhinus canicula* was not affected by hypoxic exposure, but at 12°C, O_2 uptake

decreased with falling water PO_2 , and at 17°C, P_{crit} was greatly increased to the point that the sharks were almost fully oxy-conformers (Butler and Taylor, 1975). The P_{crit} values for hypoxia-tolerant *Hemiscyllium ocellatum* and the hypoxia-sensitive *Aptychotrema rostrata* are lower than those of previously studied elasmobranch species (Piper et al., 1970; Butler and Taylor, 1975; Chan and Wong, 1977; Speers-Roesch et al., 2012a; but see Routley et al., 2002 for further discussion), and the P_{crit} for *Hemiscyllium ocellatum* is the lowest reported for any elasmobranch (Routley et al., 2002; Speers-Roesch et al., 2012a). Some species of fishes have evolved an increased capacity for O_2 -independent mechanisms of ATP production (Richards, 2009; Mandic et al., 2013), as well as tissue-specific metabolic responses to hypoxia (Speers-Roesch et al., 2012b), thus P_{crit} is not necessarily associated with tissue-level or functional hypoxia tolerance, and thus should be considered as just one of many possible measures of hypoxia tolerance (Mandic et al., 2013; Speers-Roesch et al., 2013). Nevertheless, P_{crit} is predictive of Hb- O_2 saturation and CaO_2 during hypoxia exposure in *Hemiscyllium ocellatum* and *Aptychotrema rostrata*. The lower P_{crit} of the hypoxia-tolerant *Hemiscyllium ocellatum* ($P_{crit} = 38$ mmHg) reflects the higher Hb- O_2 affinity (*in vivo* $P_{50} = 32$ mmHg) of this shark compared to the hypoxia sensitive *Aptychotrema rostrata* ($P_{crit} = 54$ mmHg; *in vivo* $P_{50} = 48$ mmHg). Thus, the hypoxia tolerance of *Hemiscyllium ocellatum*, a tropical shark that inhabits shallow coral reef environments where nocturnal hypoxia is common, is largely attributable to its enhanced O_2 transport characteristics and hypoxic cardiovascular function compared to hypoxia sensitive species (Routley et al., 2002; Nilsson and Renshaw, 2004; Speers-Roesch et al., 2012a,b).

2.4.4. SALINITY

Most elasmobranchs are marine-dwelling (stenohaline seawater), although some species are capable of entering reduced salinity or freshwater for all or part of their lives (euryhaline), and a small number of species have adapted to live entirely in freshwater (stenohaline freshwater) (Martin, 2005; Ballantyne and Fraser, 2013). Aspects of the biology and physiology, including osmoregulation and metabolism, of freshwater, marine, and euryhaline elasmobranchs were expertly reviewed by Ballantyne and Fraser (2013), and ionoregulation and acid–base balance are reviewed by Wright and Wood (Chapter 5). When euryhaline species such as the bull shark, *Carcharhinus leucas*, and the Atlantic stingray, *Dasyatis sabina*, move between seawater and freshwater important physiological reorganization occurs, including adjustments in the concentrations of solutes such as Na^+ , Cl^- , and urea (Evans et al., 2004; Ballantyne and Fraser, 2013; see also Chapter 5). The consequent fall

in urea can detrimentally affect physiological function, and urea balance may ultimately be a primary determinant of survival in dilute seawater and freshwater (Guffey and Goss, 2014). The consequent plasma dilution that occurs upon freshwater entry, owing to an osmotic water load and decreased osmolyte levels can affect blood O₂-carrying capacity and Hb-O₂ binding characteristics.

The hematological effects of acute exposure or experimental acclimation to dilute seawater vary among elasmobranch species. Transfer to reduced strength seawater had no lasting hematological effects in juvenile lemon sharks, *Negaprion brevirostris*, leopard sharks, *Triakis semifasciata*, or common stingarees, *Trygonoptera testacea*, but caused Hct and Hb concentration to fall in the dusky shark, *Carcharhinus obscurus*, and the sandbar shark, *Carcharhinus plumbeus*, although sandbar sharks did exhibit a modest osmoregulatory capacity (Goldstein et al., 1968; Cooper and Morris, 1998; Dowd et al., 2010; Pace, 2006). Goldstein and Forster (1971) reported a 20% decrease in Hct for *Leucoraja erinacea*, (= *Raja erinacea*) transferred to 50% seawater, whereas no change in Hct was observed by Forster and Goldstein (1976). Acute transfer to dilute seawater had no lasting hematological effects in Port Jackson sharks, *Heterodontus portusjacksoni*, despite plasma dilution, loss of osmolytes, and initial declines in Hct and Hb concentration (Cooper and Morris, 1998). Chronic exposure, however, reduced Hb-O₂ affinity and caused Hct to fall by around 29%, effectively decreasing blood-O₂ content, although blood PO₂, the CaO₂-CvO₂ difference, and $\dot{M}O_2$ remained unchanged and thus gas exchange was not impaired (Cooper and Morris, 2004). Although some populations of euryhaline elasmobranchs spend all or part of their lives in freshwater, they appear to remain capable of moving between freshwater and seawater environments (Piermarini and Evans, 1998; Pillans et al., 2005; Ballantyne and Fraser, 2013). *Dasyatis sabina* and *Carcharhinus leucas* captured in freshwater exhibited osmoregulatory plasticity to acclimate to full strength seawater with no significant reductions in Hct (Piermarini and Evans, 1998; Pillans et al., 2005). Hematocrit levels were also similar for *Carcharhinus leucas* captured in freshwater, euryhaline, and marine environments (Thorson et al., 1973), and stripped Hb P₅₀ values as well as the shape and position of oxygen equilibrium curves were almost identical for *Carcharhinus leucas* captured from Lake Nicaragua, the Rio San Juan, and the Caribbean Sea (Burke, 1974). In South American freshwater stingrays, *Potamotrygon* sp., gradually acclimated (\approx 2 months) to 40% seawater, plasma Na⁺ and Cl⁻ levels were elevated, but Hct remained unchanged, indicating the ability of these rays to maintain water balance. Furthermore, *Potamotrygon* Hb is insensitive to Cl⁻ (Martin et al., 1979) as is Hb from *Dasyatis sabina* (Mumm et al., 1978), which is one of the few

euryhaline elasmobranchs that can successfully complete its entire life cycle in freshwater (Johnson et al., 1996; Ballantyne and Fraser, 2013). The cownose ray, *Rhinoptera bonasus*, has been documented in salinities as low as 8‰ (Smith and Merriner, 1987) and also possesses Cl^- insensitive Hbs; however, so does the stenohaline spiny butterfly ray, *Gymnura altavela* (Scholnick and Mangum, 1991). Since all four of these rays are in the order Myliobatiformes it is unclear whether Cl^- insensitive Hbs are common to Myliobatiform rays or if this trait is beneficial for a euryhaline lifestyle (Scholnick and Mangum, 1991).

2.4.5. TEMPERATURE

Over a century ago, Barcroft and King (1909) demonstrated that rising blood temperature decreased whole blood Hb- O_2 affinity, which they proposed would benefit O_2 delivery to warm, metabolically active tissues. Krogh and Leitch (1919) were the first to note the significance of the temperature sensitivity of Hb- O_2 affinity for O_2 uptake in fishes, recognizing that blood passing through the gills equilibrates to ambient water temperature. Environmental temperature also strongly influences the metabolic rate of most fishes, and this situation is true of elasmobranchs (reviewed by Bernal et al., 2012). Consequently, rising environmental temperature can increase the metabolic demand for O_2 while simultaneously decreasing Hb- O_2 affinity and thus, blood-oxygenation at the gills of elasmobranchs.

For *Scyliorhinus canicula* acclimated to different seasonal temperatures (7, 12, and 17°C) O_2 uptake was increased at higher temperatures, which was associated with increases in heart rate, cardiac output, and ventilatory frequency (Butler and Taylor, 1975). Arterial PO_2 was similar at high and low temperatures, whereas Hct was elevated in sharks acclimated to 17°C relative to 7°C (Table 3.3), but measured blood O_2 contents were similar. The first published account of the effect of temperature on whole blood Hb- O_2 affinity of an elasmobranch was conducted by Dill et al. (1932) on blood from the winter skate, *Leucoraja ocellata* (= *Raia oscillata*), in which warming of the skate's blood predictably increased P_{50} linearly over a wide range of temperatures (0.2 to 37.5°C). Warming the blood of other elasmobranchs to ecologically relevant temperatures similarly increased P_{50} for the marbled electric ray, *Torpedo marmorata*, dusky smooth-hound, *Mustelus canis*, and the blue shark, *Prionace glauca* (Hughes, 1978; Powers et al., 1979a; D. Bernal, J. Graham, and J. Cech, unpublished data in Skomal and Bernal, 2010). Interestingly, in juvenile sandbar sharks, *Carcharhinus plumbeus*, the enthalpy ($\Delta H'$) of whole blood Hb-oxygenation appears to be temperature dependent, as is indicated by a nonlinear van't Hoff plot (Fig. 3.4A). Although the P_{50} of sandbar shark blood increases

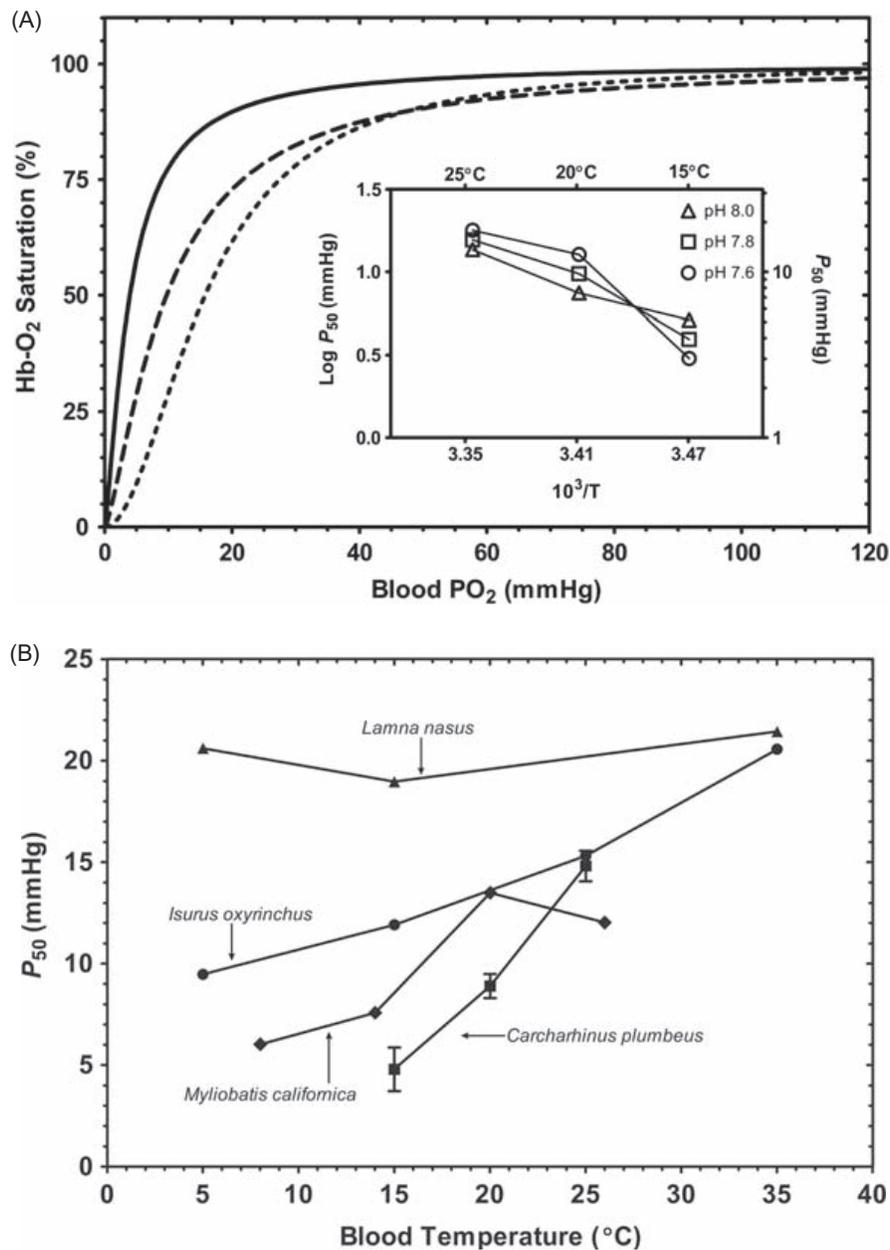


Figure 3.4. The effect of temperature on whole blood Hb-O₂ affinity of elasmobranchs. Oxygen equilibrium curves for *Carcharhinus plumbeus* at pH 7.8 and 15°C (solid line; $P_{50} = 4$), 20°C (dashed line; $P_{50} = 10$), and 25°C (dotted line; $P_{50} = 16$) are shown in panel (A) with an inset of the van't Hoff plot where $\log P_{50}$ values were interpolated from $\log P_{50}$ versus pH plots, at pH 7.6 (circles), 7.8 (squares), and 8.0 (triangles). The effect of temperature on P_{50} values for *Lamna nasus* (triangles), *Isurus oxyrinchus* (circles), *Myliobatis californica* (diamonds), and *Carcharhinus plumbeus* (squares) are shown in panel (B). P_{50} values for *Lamna nasus* and *Isurus oxyrinchus* were interpolated from Hill's plots in Andersen et al. (1973), *Myliobatis californica* P_{50} 's are from Hopkins and Cech (1994a), and the data for *Carcharhinus plumbeus* are from unpublished experiments by two of the authors (P.R. Morrison, T.S. Harter, R. Brill, and C.J. Brauner, unpublished data).

with an increase in temperature between 15, 20, and 25°C [which is within the range of water temperatures experienced by juvenile sandbar sharks throughout a year (Conrath and Musick, 2008)] values for $\Delta H'$ become more endothermic (less negative) as temperature increases and as pH falls at 25°C (P. R. Morrison, T. S. Harter, R. Brill, and C. J. Brauner, unpublished data). The bat eagle ray, *Myliobatis californica*, also exhibits an atypical blood temperature sensitivity where an increase in temperature from 14 to 20°C ($PCO_2 \approx 0.2$ mmHg) increased whole blood P_{50} , but changes in temperature above (20 to 26°C) or below (8 to 14°C) that range had no effect on P_{50} (Fig. 3.4 and Table 3.3; Hopkins and Cech, 1994a). The reduced temperature sensitivity of Hb-oxygenation occurs at the extreme high and low temperatures that bat eagle rays normally encounter (Hopkins and Cech, 1994a), which represent the temperatures at which resting O_2 consumption rates (i.e., Q_{10}) are least sensitive to temperature ($Q_{10} = 1.85$) (Hopkins and Cech, 1994b). The temperature dependency of the heat of Hb-oxygenation in *Carcharinus plumbeus* and *Myliobatis californica* may be related to the eurythermal life history of these two elasmobranchs, but any adaptive or physiological significance is not clear. Alternatively, Fago et al. (1997) suggested that the temperature dependency of $\Delta H'$ may not be a specific adaptive mechanism, but may result from variation in the oxygenation-dependent release of allosteric effectors, and may thus be a characteristic of Hbs with large Bohr effects. The temperature dependency of $\Delta H'$ for other stenothermal and eurythermal elasmobranchs has not been investigated and is clearly an area worthy of further investigation

2.4.6. REGIONALLY HETEROTHERMIC ELASMOBRANCHS

Sharks in the family Lamnidae (i.e., *Isurus oxyrinchus*, *Isurus paucus*, *Carcharodon carcharias*, *Lamna nasus*, and *Lamna ditropis*) and the common thresher shark, *Alopias vulpinus*, have evolved the ability to retain metabolic heat within the circulatory system so as to maintain select tissues at temperatures warmer than the ambient water temperature, thus these sharks are functionally regional heterotherms (often described as regional endotherms) (see Bernal and Lowe, 2015). In most fishes, the red myotomal muscles (RM) that power aerobic locomotion are positioned subcutaneously (i.e., laterally) along the length of the body, which causes metabolic heat produced by the RM to be quickly lost to the environment through conduction across the body wall, as well as by convective transfer through the gill circulation (Stevens and Sutterlin, 1976; Brill et al., 1994). This is not the case in the lamnid sharks and *Alopias vulpinus*, in which the RM is located medially and more anteriorly in the body, very close to the vertebral column (Carey and Teal, 1969; Carey et al., 1985; Sepulveda, 2005; Bernal et al., 2012). Regionally heterothermic sharks must sustain aerobic locomotion

to ventilate their gills (i.e., ram ventilation; see Chapter 2), so the RM provides a constant source of heat that is insulated from the environment by the white muscle, and retained within the circulatory system by vascular counter-current heat exchangers (*retia mirabilia*), which transfer heat from the warm venous blood to the cool arterial blood that has thermally equilibrated with ambient water during passage through the gills (Burne, 1924; Carey et al., 1985; Bernal et al., 2001; Patterson et al., 2011). Furthermore, lamnid sharks also possess a suprahepatic *rete*, which warms the viscera and liver by retaining the heat produced during digestion and assimilation, as well as an orbital *rete* that warms the brain and eyes of these sharks (Burne, 1924; Carey et al., 1981; Block and Carey, 1985; reviewed by Bernal et al., 2001). *Alopias vulpinus* also has a *rete* associated with the viscera; there are RM associated *retia* in the pectoral fins of the Chilean devil ray, *Mobula tarapacana*; and orbital *retia* are present in some species of mobulid rays and the bigeye thresher shark, *Alopias superciliosus* (Alexander, 1995; Alexander, 1996; Fudge and Stevens, 1996; Weng and Block, 2004); however, it has yet to be confirmed if these vascular counter-current exchangers have a thermoconserving function (see Bernal and Lowe, 2015).

The evolution of heat exchanging *retia* associated with the RM of regionally heterothermic sharks requires their systemic circulation to be very different from the typical central circulatory system of most elasmobranchs (i.e., ectothermic elasmobranchs) (see Bernal and Lowe, 2015). In most fishes, blood flow to and from the systemic circulation is through centrally located vessels (i.e., the dorsal aorta, and post-cardinal vein) that run ventral to the vertebral column. This scenario is quite different in regionally heterothermic sharks with internalised RM, where most of the systemic blood flow is through large lateral subcutaneous vessels that are located just under the skin on both sides of the body (Burne, 1924; Carey and Teal, 1969; Bernal et al., 2001; Patterson et al., 2011). The lateral arteries, which arise from the dorsal aorta in *Alopias vulpinus* and from the efferent branchials in the lamnid sharks (see Bernal and Lowe, 2015), distribute cool, oxygenated blood through the RM *retia* where it is rapidly warmed. For example, in the salmon shark, *Lamna ditropis*, the RM temperature may be as much as 15–20°C warmer than the peripheral tissues and ambient water temperature (Anderson et al., 2001; Goldman et al., 2004; Bernal et al., 2005), which causes the blood to flow through a steep temperature gradient. When blood enters the warmer tissues through the *retia* it is subjected to what has been referred to as a “closed-system” temperature change because the increase in temperature can have a large effect on blood PO_2 and PCO_2 , but the content of blood gases stays relatively constant (Brill and Bushnell, 1991). Furthermore,

regionally-heterothermic sharks, and other pelagic elasmobranchs, may make long-distance latitudinal migrations (e.g., Weng et al., 2005; Block et al., 2011; Saunders et al., 2011) and/or deep vertical sojourns throughout the water column (e.g., Carey and Scharold, 1990; Weng and Block, 2004; Nasby-Lucas et al., 2009; Abascal et al., 2011; Carlisle et al., 2011; Thorrold et al., 2014) exposing the blood flowing through their gills to changing water temperature. Because blood in the gill equilibrates with water temperature and gas tensions, it is considered an “open system.” These rapid open- and closed-system temperature changes can potentially affect Hb-O₂ affinity and blood-O₂ transport, but the limited evidence available indicates that blood of lamnid sharks is largely unaffected by temperature, unlike the blood of most fishes.

Warming the blood of regionally heterothermic sharks either decreases or slightly increases P_{50} , as was first documented in sharks by Andersen et al. (1973) for the porbeagle, *Lamna nasus*, and shortfin mako, *Isurus oxyrinchus*. Andersen and coworkers coupled studies on Hb solutions (i.e., CO replacement) with whole-blood-oxygen equilibria, but displayed only whole blood data from 20% to 80% Hb-O₂ saturation (i.e., Hill Plots). For *Lamna nasus* blood, Hill plots that correspond to 5°C, 15°C, and 35°C open-system temperature changes cross over above 30% Hb-O₂ saturation, evidence of reverse temperature-dependent blood oxygenation (Andersen et al., 1973). The saturation dependency of the reverse temperature sensitivity of *Lamna nasus* blood has been similarly observed in the blood of bluefin tuna, *Thunnus thynnus* (Carey and Gibson, 1983; Ikeda-Saito et al., 1983), and likely results from the endothermic release of allosteric effectors late in the oxygenation process (Ikeda-Saito et al., 1983; Weber and Campbell, 2011). Open-system temperature changes from 5°C to 35°C had little effect on the slope of Hill plots for *Isurus oxyrinchus* blood, but Hb-O₂ affinity was reduced (Andersen et al., 1973) but not nearly to the same extent as that observed in two ectothermic sharks, the sandbar shark, *Carcharhinus plumbeus* (Fig. 3.4A and B), and the blue shark, *Prionace glauca* (D. Bernal, J. Graham, and J. Cech, unpublished data in Skomal and Bernal, 2010). Moreover, Bernal, Cech, and Graham observed that whole blood P_{50} values for *Isurus oxyrinchus* were almost invariant to open system temperature changes indicative of temperature independent blood oxygenation, whereas closed system temperature changes slightly decreased P_{50} , a reverse temperature effect (D. Bernal, J. Graham, and J. Cech, unpublished data in Skomal and Bernal, 2010).

Fundamentally, reduced and reverse temperature-dependent blood oxygenation seems counterintuitive for regionally heterothermic fishes because warming of the blood will increase Hb-O₂ affinity, effectively decreasing O₂ diffusion between the blood and a mitochondrion in the warm

tissues (Bushnell and Jones, 1994). In tuna, closed-system warming of the blood predictably increases PCO_2 and decreases pHe, but the interacting effects of simultaneous changes to blood temperature and pH differentially influence P_{50} values and Bohr coefficients among tuna species (Cech et al., 1984; Brill and Bushnell, 1991; Lowe et al., 2000; Brill and Bushnell, 2006). Despite species-specific responses to closed-system warming of the blood, oxygen delivery to the warm, aerobically active tissues of tunas may be enhanced by the large Bohr effect that is generally associated with a Root effect (Lowe et al., 1998; Brill and Bushnell, 2006; Randall et al., 2014). This scenario has recently been proposed to occur in a salmonid by short-circuiting of RBC pHi regulation by plasma accessible carbonic anhydrase (Rummer and Brauner, 2011; Rummer et al., 2013). For *Isurus oxyrinchus*, O_2 offloading in the capillaries of the warm tissues does not seem to be enhanced by increased blood temperature or by the Bohr effect (Andersen et al., 1973; Wells and Davie, 1985; D. Bernal, J. Graham, and J. Cech, unpublished data in Skomal and Bernal, 2010). It may be that the high Hb concentrations and Hct values measured in *Isurus oxyrinchus* (Table 3.3) maintain the high rates of O_2 delivery needed to match high O_2 consumption rates (Sepulveda et al., 2007), which will maintain a steady production of metabolic heat. The steady consumption of oxygen will maintain a steep PO_2 gradient between the blood and tissues, causing O_2 to dissociate from Hb and travel down its partial pressure gradient. This PO_2 gradient may be sustained by high tissue concentrations of myoglobin measured in regionally heterothermic sharks (Bernal et al., 2003), which will accelerate shuttling of O_2 between the blood and the mitochondria.

The remarkable functional convergence among regionally heterothermic vertebrates including fishes, birds, and mammals for Hb that binds O_2 independent of temperature likely resulted from similar selective pressures to match tissue O_2 supply with demand in spite of steep internal temperature gradients (Weber and Campbell, 2011). The functional significance of reduced and reverse temperature dependent Hb-oxygenation in regionally heterothermic fishes has been proposed to increase the range of water temperatures a species can exploit without compromising O_2 uptake at the gills (Rossi-Fanelli and Antonini, 1960). It has also been proposed to prevent excessive O_2 offloading as blood is rapidly warmed in the heat exchanging *retia* (Graham, 1973; Bernal et al., 2009), while maintaining O_2 delivery to the tissues and organs that are at or near ambient water temperature (Clark et al., 2008). Some researchers have suggested that the O_2 offloading that would occur if P_{50} was increased by warming of the blood in a *rete* would increase blood PO_2 , which would cause the formation of a diffusion gradient for O_2 between the arterial and venous systems of a *rete* (Carey and Gibson, 1977; Dickinson and Gibson, 1981; Larsen et al., 2003);

however, the size of the vessels, and the distances between them are likely too large for substantial O₂ diffusion to occur in the heat exchanging *retia* of regionally heterothermic fishes (Stevens et al., 1974; Carey et al., 1985). Importantly, reverse temperature dependent Hb-oxygenation probably reduces oxygenation-linked heat loss via the circulatory system by “swapping” the normal exothermic and endothermic enthalpies of oxygenation at the gill and tissue compartments, respectively (Weber and Fago, 2004; Weber et al., 2010). Intriguingly, reduced and reverse temperature-dependent blood oxygenation also occurs in the chub mackerel, *Scomber japonicas*, an ectothermic fish closely related to the tunas (Clark et al., 2010). Clark et al. (2010) speculated that reduced and reversed Hb temperature effects may have preceded the evolution of regional heterothermy in tunas, in which case this trait may be an exaptation for regional heterothermy in fishes, most likely for its heat conservation benefits by reducing oxygenation-linked heat loss (Weber et al., 2010).

It is not known whether the lamnid sharks’ close relatives in the order Lamniformes, or suspected regionally heterotherms also possess Hb with a reduced or reverse temperature dependency. However, blood oxygenation in the bat eagle ray, *Myliobatis californica*, a close relative of the mobulid rays that are suspected to be regionally heterotherms, shows a reduced temperature dependency at the extreme low and high temperatures that this species encounters (Fig. 3.4B; Hopkins and Cech, 1994a). The temperature dependency of Hb-oxygenation needs to be further investigated in the Lamnid sharks, as well as in *Alopias vulpinus*, suspected regional heterotherms (i.e., *Mobula tarapacana*, and *Alopias superciliosus*), and ectothermic sharks in the order Lamniformes to investigate the evolution of this trait as it relates to regional heterothermy and the thermal niche of each species.

3. TRANSPORT AND ELIMINATION OF CARBON DIOXIDE

In most vertebrates, including most teleosts, the RBC plays a predominant role in the transport and elimination of CO₂, even though the majority of total CO₂ is carried in the plasma as HCO₃⁻ ions (e.g., see reviews by Perry, 1986; Randall and Val, 1995; Henry and Heming, 1998; Tufts and Perry, 1998; Swenson, 2000; Geers and Gros, 2000; Henry and Swenson, 2000; Tufts et al., 2003; Evans et al., 2005; Esbaugh and Tufts, 2006b). From its tissue site of production, CO₂ diffuses into the RBC where it is hydrated to H⁺ and HCO₃⁻ in a reaction catalyzed by RBC cytosolic CA (Meldrum and Roughton, 1933). Bicarbonate then exits the RBC in exchange for Cl⁻ via the band 3 anion exchanger located on the RBC membrane (Romano and Passow, 1984; Hubner et al., 1992) and protons

are buffered by Hb, with removal of these end-products of the hydration reaction serving to enhance CO₂ loading into the blood. The process is reversed when the blood reaches the gas exchange organ. As molecular CO₂ diffuses out of the RBC and across the gas exchange surface according to its partial pressure gradient, HCO₃⁻ moves from the plasma into the RBC via band 3 and is dehydrated to CO₂ in a reaction also catalyzed by RBC cytosolic CA. The central role of the RBC in this model is assured by its high abundance of the two key metalloproteins, CA and Hb (Henry and Swenson, 2000), as well as the presence of the rapid anion exchanger, and is supported experimentally by the impairment of CO₂ excretion observed in teleost fish when Hct is lowered below ~5% (Wood et al., 1982; Gilmour and Perry, 1996; Gilmour and MacNeill, 2003). By contrast, CO₂ excretion in the dogfish *Squalus suckleyi* (= *Squalus acanthias*) was unaffected when Hct was lowered to 5% by blood withdrawal coupled with volume replacement by saline (Gilmour and Perry, 2004). This observation, together with findings of the presence of extracellular CA in dogfish gills and blood, indicate that CO₂ excretion in the dogfish *Squalus suckleyi*, and potentially elasmobranchs in general, does not follow the typical model of CO₂ excretion in teleosts described above (reviewed by Gilmour and Perry, 2010). The sections that follow will first discuss transport of CO₂ in the blood of elasmobranchs and then focus on CO₂ excretion, with emphasis on the tissue distribution of CA isoforms and their likely roles in CO₂ excretion. The latter discussion will, of necessity, revolve around the dogfish *Squalus suckleyi* because this is the species for which data are available, but evidence from other species will be presented where possible.

3.1. Carbon Dioxide Transport in Blood

Carbon dioxide is transported in the blood primarily as HCO₃⁻ with smaller amounts transported as physically dissolved CO₂ and as carbamino CO₂ (i.e., bound to Hb and plasma proteins). Since the α-amino groups of the α-chains of most sequenced elasmobranch Hbs are not acetylated (Fig. 3.1), CO₂ transport as carbamino CO₂ bound to Hb may be relatively more important in elasmobranchs than it is in teleosts. The CO₂ combining curve describes the relationship between total CO₂ and blood PCO₂, and thus the CO₂ capacitance of the blood. Arterial and venous PCO₂ are generally low in the blood of elasmobranchs (Table 3.3), and therefore elasmobranchs tend to work on the steep portion of the combining curve resulting in a high CO₂ capacitance; for example, CO₂ capacitance in the blood of *Scyliorhinus stellaris* is approximately 50 to 70 times greater than O₂ capacitance (Piiper and Baumgarten-Schumann, 1968). In most elasmobranchs studied to date, the CO₂ capacitance of separated plasma

is similar to that of whole blood and true plasma, whereas separated plasma from teleosts generally has a substantially lower CO₂ capacitance than whole blood (Table 3.3; reviewed in Tufts and Perry, 1998). ‘True’ and ‘separated’ plasma refer, respectively, to blood that has been equilibrated with CO₂ and then centrifuged to yield plasma versus plasma that has been separated from blood and subsequently equilibrated with CO₂ in isolation, and therefore the similarity of CO₂ capacitances among separated plasma, whole blood, and true plasma in elasmobranchs implies significant CO₂ capacitance in the plasma itself. The buffer capacity of blood is primarily a function of the bicarbonate buffering system, together with nonbicarbonate buffers that include phosphates, and most importantly, the imidazole side chain of histidine residues in Hb and plasma proteins. Whereas the buffer capacity of separated plasma from teleosts lacks significant contributions from plasma proteins, and thus has a lower buffer capacity than whole blood [typically 20–40% of the whole blood value; (Gilmour et al., 2002)], in which buffer capacity is dominated by the contribution of Hb, the buffer capacity of separated plasma from elasmobranch blood can reach 60–70% of that of whole blood (Table 3.3). Unexpectedly, the buffer capacity of separated plasma from *Scyliorhinus stellaris* was low (30% of the whole blood value), which was inconsistent with the high CO₂ capacitance exhibited by separated plasma in this shark (Tufts and Perry, 1998); carbamino CO₂ formation with plasma proteins could explain this discrepancy (Tufts and Perry, 1998). High buffer capacity in the plasma compartment of elasmobranch blood likely plays an important role in CO₂ excretion by providing protons for HCO₃⁻ dehydration in the plasma (see Section 3.2.3).

In the presence of a Haldane effect, deoxygenated blood contains more CO₂ than oxygenated blood (Christiansen et al., 1914); proton binding to oxy-labile sites on Hb enhances CO₂ hydration in the RBC, leading to greater loading of CO₂ into the blood. In elasmobranchs, the Haldane effect generally is considered to be small and to play a negligible role in blood CO₂ transport (Wood et al., 1994). Although small but distinct Haldane effects have been reported for *Squalus acanthias* (Weber et al., 1983a) and *Raja clavata* (= *Raia clavata*) (Hughes and Wood, 1974), the Haldane effect has been concluded to be functionally absent from the blood of *Squalus suckleyi* (Lenfant and Johansen, 1966), *Leucoraja ocellata* (= *Raia oscillata*) (Dill et al., 1932), *Scyliorhinus stellaris*, *Mustelus mustelus*, *Torpedo torpedo* (= *Torpedo ocellata*) (Albers and Pleschka, 1967), *Mustelus canis* (Ferguson et al., 1938), *Triakis semifasciata* (Lai et al., 1990), and *Scyliorhinus canicula* (Pleschka et al., 1970; Wood et al., 1994). However, Hb buffer values are high in elasmobranchs and high Hb buffer capacity, like the Haldane effect, can facilitate CO₂ transport in the blood by removing the protons generated by CO₂ hydration in the RBC.

The content and tension of CO₂ in the blood also exhibit temperature dependency. [Albers and Pleschka \(1967\)](#) noted that, at temperatures from 9 to 25°C and *PCO*₂ from 2 to 10 mmHg, the relative change of total CO₂ in the blood of *Scyliorhinus stellaris* and *Torpedo torpedo* (= *Torpedo ocellata*) was 2.5% per °C, but buffer values for true plasma and separated plasma were not affected by temperature changes. The solubility of CO₂ in plasma decreases as temperature increases ([Pleschka and Wittenbrock, 1971](#)), so accordingly at constant total CO₂ blood *PCO*₂ rises with increasing temperature. Because water-breathing species have a very low blood *PCO*₂ (e.g., see [Perry and Gilmour, 2006](#)), arterial pH is predominantly regulated by adjusting HCO₃⁻ concentration in exchange for Cl⁻ rather than by altering *PCO*₂ via changes in ventilation ([Heisler, 1988](#)). Heisler and collaborators provided evidence for this phenomenon in *Scyliorhinus stellaris*, in which the regulation of extracellular and tissue pH with environmental temperature was shown to be virtually independent of arterial *PCO*₂ but dependent upon changes in HCO₃⁻ concentrations ([Heisler et al., 1980](#); [Heisler, 1988](#)).

Exercise is expected to influence the production of CO₂ and hence blood CO₂ content and tension. However, several reports suggest that the impact of exercise on arterial *PCO*₂ (*PaCO*₂) values in elasmobranchs may be relatively small. For example, in *Triakis semifasciata* forced to sustain moderately intense aerobic swimming activity, *PaCO*₂ increased significantly by 29% but returned to resting conditions within an hour following the cessation of swimming, and neither arterial CO₂ content nor arterial pH changed significantly ([Lai et al., 1990](#)). Similarly, *PaCO*₂ was not significantly elevated by exhaustive exercise in *Squalus suckleyi* (= *Squalus acanthias*) although an acidosis of metabolic origin was observed ([Richards et al., 2003](#)). By contrast, *PaCO*₂ nearly doubled immediately after exhaustive exercise in *Scyliorhinus stellaris*, with values returning to control levels within an hour ([Piper et al., 1972](#)), and *PaCO*₂ remained elevated for several hours when *Scyliorhinus stellaris* were electrically stimulated to exhaustion ([Holeton and Heisler, 1983](#)). The presence of plasma-accessible CA in the gill circulation (see [Section 3.2.2](#)), which will catalyze HCO₃⁻ dehydration within the plasma, which facilitates CO₂ clearance from the plasma at the gill, has been suggested as an explanation of the muted impact of exercise on arterial *PCO*₂ in at least some exercising elasmobranchs ([Richards et al., 2003](#)) and is elaborated upon in more detail below.

3.2. Carbon Dioxide Excretion

The model of CO₂ excretion developed for *Squalus suckleyi* [formerly *Squalus acanthias* ([Ebert et al., 2010](#))] differs from that for most other

vertebrates in proposing two sites of CA-catalyzed HCO_3^- dehydration when blood reaches the gill; namely, plasma and RBCs (reviewed by Gilmour and Perry, 2010). Dehydration of HCO_3^- in RBCs is catalyzed by cytosolic CA, whereas plasma HCO_3^- dehydration is catalyzed by extracellular CA, specifically branchial membrane-bound CA. It is therefore useful to first consider the diversity and distribution of CA isoforms in the blood and gill of elasmobranch fish (for a broader review of fish CA isoforms, see Gilmour and Perry, 2009) before examining the experimental support for this model of CO_2 excretion. It is important to reiterate that this model has also been developed on the basis of experimental evidence gathered in *Squalus suckleyi* and has yet to be extended to other elasmobranch species.

3.2.1. THE EVOLUTION OF CA ISOFORMS

Carbonic anhydrase is the zinc metalloenzyme that catalyzes the reversible reactions of CO_2 and water. Vertebrates express multiple CA isoforms, including several CA-related proteins that lack catalytic activity (Tashian et al., 2000; Esbaugh and Tufts, 2007; Lin et al., 2008). Among the isoforms with catalytic activity, both intracellular and extracellular enzymes occur (see reviews by Chegwiddden and Carter, 1999; Hewett-Emmett, 2000; Hilvo et al., 2008; Gilmour and Perry, 2009). The extracellular CAs include the secreted isoform CA VI, several isoforms that are expressed as single-pass transmembrane proteins (CAs IX, XII, and XIV), and two isoforms, CAs IV and XV, that are anchored to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage. Representatives of all of these isoforms have been identified in teleosts (Lin et al., 2008) and the holocephalan *Callorhinchus milii* (<http://esharkgenome.imcb.a-star.edu.sg/>) as well as mammals, and the diversity of CA IV-like isoforms appears to be higher in teleosts than in mammals (Lin et al., 2008). Data for elasmobranchs, however, are largely lacking.

The intracellular CAs include one or two mitochondrial isoforms (CA V) as well as cytosolic isoforms, with the main cytosolic isoforms differing between fish (agnathans, elasmobranchs, and teleosts) and mammals (reviewed by Gilmour and Perry, 2009). The cytosolic isoform CA VII appears in mammals as well as both teleosts and elasmobranchs. However, whereas mammals express a cluster (CAs I, II, III, XIII) of closely-related cytosolic CA isoforms, the existing phylogenetic evidence, which admittedly is scanty, suggests that fish retained the ancestral state of a single, high activity CA isoform until the appearance of the teleosts. In the teleost line, a whole genome duplication gave rise to two closely-related cytosolic isoforms, a higher-activity form that is expressed predominantly in the blood, and a broadly-distributed isoform of slightly lower activity (Rahim et al., 1988; Esbaugh et al., 2004, 2005; Lin et al., 2008). Data for

elasmobranchs appear to be limited to sequences for CA VII for *Squalus acanthias* (GenBank accession CX196604) and a RBC cytosolic CA sequence for *Squalus suckleyi* (= *Squalus acanthias*) (Gilmour et al., 2007), making it difficult to draw conclusions about the evolution of cytosolic CA isoforms in elasmobranchs. From this diversity of CA enzymes, cytosolic isoforms found in the RBC and extracellular CA IV are of particular interest with respect to CO₂ excretion.

3.2.2. CARBONIC ANHYDRASE ISOFORMS INVOLVED IN CO₂ EXCRETION IN ELASMOBRANCHS

Carbonic anhydrase activity is associated with both the plasma and RBC of elasmobranch blood. Analysis of separated plasma from the dogfish *Squalus suckleyi* (= *Squalus acanthias*) (Gilmour et al., 1997; Henry et al., 1997) and *Scyliorhinus canicula* (Wood et al., 1994), as well as the skate *Raja rhina* (Gilmour et al., 2002) in the presence and absence of the CA inhibitor acetazolamide revealed the presence of measureable CA activity. The source of this plasma CA activity may be lysed RBCs (Henry et al., 1997), although this hypothesis requires confirmation through direct comparison of plasma and RBC CA sequences. The plasma of elasmobranchs examined to date lacks an endogenous CA inhibitor (Henry et al., 1997; Gilmour et al., 2002), and therefore CA released during the natural turnover and lysis of RBCs could potentially remain active in the plasma. However, the low level of activity detected in plasma limits the catalytic potential of this CA source; Henry et al. (1997) estimated that plasma CA activity in *Squalus suckleyi* was only 0.02% of that in an equivalent volume of RBCs.

Although RBC CA activity greatly exceeds that of plasma, the available data, while sparse, suggest that the RBCs of elasmobranchs exhibit low CA activity in comparison to those of other vertebrates in general and teleosts in particular. For example, CA activity in the RBCs of the dogfish *Squalus acanthias* and *Squalus suckleyi* was found to be 6 to 14-fold lower than that in rainbow trout, flounder, or goosefish (Maren et al., 1980; Henry et al., 1997). Low RBC CA activity appears to reflect both low concentrations of CA in elasmobranch RBCs and low catalytic activity of the elasmobranch RBC CA isoform. In a direct comparison, Swenson (1979) reported that the concentration of CA in the RBCs of *Squalus acanthias* was only 19–28% of that in RBCs from flounder or goosefish. An examination of RBC CA concentrations reported in different studies revealed similar trends; 0.024 mmol L⁻¹ for *Squalus acanthias* (Maren et al., 1980; Swenson and Maren, 1987) versus 1.1 mmol L⁻¹ for rainbow trout (Gervais and Tufts, 1999). On top of low levels of CA in elasmobranch RBCs, the turnover numbers or k_{cat} values for the catalytic activity of elasmobranch RBC CAs (0.5 to 2.5×10^{-4} s⁻¹ for *Squalus acanthias*, *Carcharhinus leucas*, and

Galeocerdo cuvieri; (Maynard and Coleman, 1971; Maren et al., 1980) are at least an order of magnitude lower than teleost values (25 to $70 \times 10^{-4} \text{ s}^{-1}$; Maren et al., 1980). The relatively low catalytic activity of elasmobranch RBC CA may result from the molecular structure of the active site of the enzyme. When the active site of a CA isoform cloned from the blood of the dogfish *Squalus suckleyi* (= *Squalus acanthias*) was analyzed, a serine residue was found to have been substituted for a histidine residue (His-64) that acts as a proton shuttle (Gilmour et al., 2007; Gilmour and Perry, 2010). The proton shuttle is a key component of high activity CA isoforms because it is the rate-limiting step in the catalytic mechanism of CA, serving to transfer a proton from the active site of the enzyme to the environment so as to regenerate the active form of the enzyme (reviewed by Lindskog and Silverman, 2000; Pastorekova et al., 2004). In mammalian CA isoforms, replacement of His-64 with amino acids that cannot transfer protons greatly reduces enzyme activity (Tu et al., 1989; Lindskog and Silverman, 2000; Stams and Christianson, 2000), and the substitution of His-64 by lysine in CA III is thought to account at least in part for the low activity of this isoform (Jewell et al., 1991). Similarly, the substitution of serine for His-64 in the RBC CA of *Squalus suckleyi* would be expected to result in low catalytic activity for this enzyme. Whether RBC CA isoforms from other elasmobranchs may also lack an efficient proton shuttle remains to be determined; sequence data for elasmobranch RBC CAs together with measurements of RBC CA concentration and catalytic activity are sorely needed. A partial sequence for tiger shark, *Galeocerdo cuvieri*, indicates RBC CA exists (Bergenheim and Carlsson, 1990), but sequence in the vicinity of His-64 is lacking. Interestingly, in the recently available sequence for RBC CA of the holocephalan *Callorhinchus milii* (GenBank accession AFM88204.1), His-64 is occupied by a histidine residue, indicating that the substitution of His-64 in *Squalus suckleyi* may be specific to elasmobranchs, if this substitution is indeed present in other elasmobranchs.

As in teleosts (Sobotka and Kann, 1941; Maren, 1967), CA is abundant within the gill of the elasmobranchs that have been examined [*Squalus acanthias*, (Henry et al., 1997; Gilmour et al., 2001); *Triakis semifasciatus*, (Conley and Mallatt, 1988); *Raja rhina*, (Gilmour et al., 2002)], with the majority of this branchial CA activity being cytosolic (Henry et al., 1997; Gilmour et al., 2002). Studies using histochemical staining or heterologous antibodies suggest that cytosolic CA is found in most cell types of the branchial epithelium, including the distinct populations of Na^+/K^+ -ATPase-rich and V-type H^+ -ATPase-rich cells specialized for active ion transport and acid–base regulation (Conley and Mallatt, 1988; Wilson et al., 2000; Tresguerres et al., 2007). Branchial cytosolic CA is thought to contribute to ionic and acid–base regulation in elasmobranchs by catalyzing the hydration

of CO₂ within gill epithelial cells to provide H⁺ and HCO₃⁻ for use as counter-ions by ion transport proteins (reviewed by Gilmour and Perry, 2009). Experimental evidence to support this role for cytosolic CA has been obtained from the reduction of ion fluxes (Payan, 1973) or the attenuation of recovery from an acid–base challenge (Hodler et al., 1955; Swenson and Maren, 1987; Tresguerres et al., 2007) that occurred following CA inhibition. In addition, cytosolic CA has been implicated in a base-sensing mechanism in gill epithelial cells of the dogfish *Squalus suckleyi* (= *Squalus acanthias*) (Tresguerres et al., 2007, 2010); reviewed by (Gilmour, 2012; Tresguerres et al., 2014). As in teleosts, branchial cytosolic CA does not contribute to CO₂ excretion because it is inaccessible to HCO₃⁻ in the plasma (Maren, 1967). The identity of the cytosolic CA isoform of the elasmobranch gill has yet to be determined. Interestingly, dogfish (*Squalus suckleyi*) gill cytosolic CA was much more sensitive to sulphonamide inhibitors than was dogfish RBC CA (Gilmour et al., 2001), which suggests that different CA isoforms are present in these two locations.

In addition to branchial cytosolic CA, membrane-associated CA also appears to be present in the gill of at least some elasmobranchs [*Squalus suckleyi* (= *Squalus acanthias*), (Gilmour et al., 2001, 2007); *Raja rhina* (Gilmour et al., 2002)], and while this membrane-associated CA constitutes only a small fraction of the total branchial CA activity (Henry et al., 1997; Gilmour et al., 2001, 2002), it may play a substantial role in CO₂ excretion (see Section 3.2.3). Molecular, biochemical, and physiological evidence suggests that the membrane-associated CA isoform is CA IV (reviewed by Gilmour and Perry, 2010). Phylogenetic analyses of a CA cloned from the gill of *Squalus suckleyi* (= *Squalus acanthias*) supported its identification as a type IV isoform. The sequence showed high similarity to mammalian and teleost fish CA IV sequences as well as molecular markers typical of CA IV, including a leader signal peptide for membrane targeting, a carboxy-terminal hydrophobic domain that is cleaved to allow attachment of the GPI anchor to a highly conserved serine residue, and cysteine residues that were predicted to form disulphide bridges (Gilmour et al., 2007). Biochemical support came from the observation that the membrane-associated CA activities of *Squalus suckleyi* (Gilmour et al., 2001) and *Raja rhina* (Gilmour et al., 2002) gills could be released from their membrane association by treatment with phosphatidylinositol phospholipase-C (PI-PLC), an enzyme that cleaves GPI linkages. In addition, this CA activity was resistant to inhibition by SDS (Gilmour et al., 2002), which reflects the stabilizing presence of disulphide bridges (Whitney and Briggler, 1982; Waheed et al., 1996). Using an *in situ* saline-perfused gill preparation from *Squalus suckleyi* (= *Squalus acanthias*), Wilson et al. (2000) reported that addition of the CA inhibitor acetazolamide produced a pH disequilibrium in

the outflowing perfusate that was not present under control conditions, physiological evidence that branchial CA activity was available to catalyze perfusate CO₂ reactions. In *Squalus suckleyi*, CA IV was localized using *in situ* hybridization and immunohistochemistry to the plasma membranes of pillar cells, the cells that line the blood space of the gill, a location in which it would be available to catalyze plasma CO₂ reactions (Gilmour et al., 2007). The presence in *Squalus suckleyi*, and possibly other elasmobranchs, of CA IV localized to gill pillar cell membranes is consistent with the capillary endothelial location of CA IV in tetrapod lungs (Whitney and Briggie, 1982; Zhu and Sly, 1990; Waheed et al., 1992; Stabenau and Heming, 2003), and contrasts with the absence of plasma-accessible CA IV from the gill of teleosts (reviewed by Gilmour and Perry, 2009). It is the presence of branchial CA IV in elasmobranchs, in conjunction with their relatively low RBC CA activity that has given rise to the elasmobranch model of CO₂ excretion (Fig. 3.5).

3.2.3. THE ELASMOBRANCH MODEL OF CO₂ EXCRETION

The elasmobranch model of CO₂ excretion posits dual reliance on RBC and plasma dehydration of HCO₃⁻ as blood passes through the gill. As in the classic model of CO₂ excretion, HCO₃⁻ carried in the plasma can enter the RBC via the band 3 anion exchanger (Obaid et al., 1979) and be dehydrated to molecular CO₂ in the presence of RBC CA, with protons being provided through the buffering action of Hb. At the same time, however, HCO₃⁻ ions can be dehydrated to molecular CO₂ in the plasma, in a reaction catalyzed by branchial CA IV, with protons being provided through the high buffering value of plasma proteins. Experimental evidence to support a substantial contribution of plasma HCO₃⁻ dehydration to CO₂ excretion has come from measurements of HCO₃⁻ clearance from the blood during passage through the gill, which was significantly, substantially (30–60%) reduced by treatment of *Squalus suckleyi* with low doses of the CA inhibitor benzolamide (Gilmour et al., 2001). The impairment of HCO₃⁻ clearance was accompanied by a significant increase in arterial PCO₂ (Gilmour et al., 2001). Benzolamide permeates cell membranes slowly, so low doses of the drug for short periods of time provide a method of inhibiting extracellular CA activity without significant inhibition of cytosolic CA isoforms (see Supuran and Scozzafava, 2004; Gilmour and Perry, 2010). As noted above, CO₂ excretion in *Squalus suckleyi* was not impaired by reducing Hct *in vivo* to 5% (Gilmour and Perry, 2004), nor did treatment of *Squalus suckleyi* with the anion exchange inhibitor 4,4-diisothiocyanostilbene-2,2-disulphonic acid (DIDS) significantly affect arterial PCO₂ or HCO₃⁻ clearance at the gill (Gilmour et al., 1997, 2001). Anion exchange is considered to be the rate-limiting step in HCO₃⁻

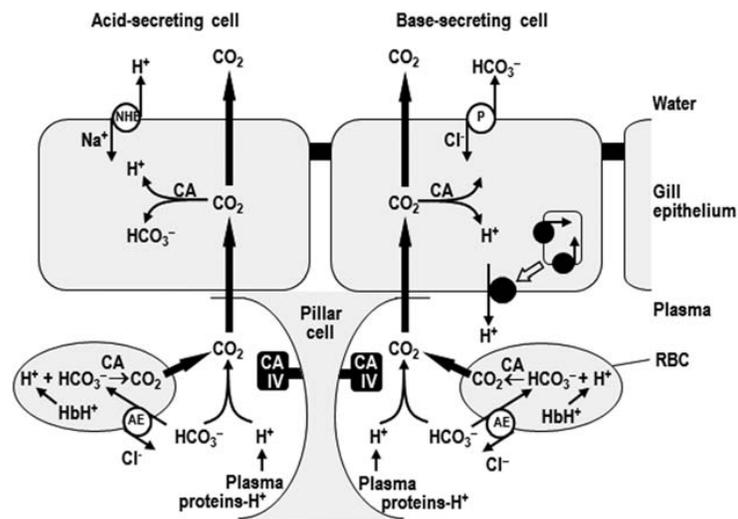


Figure 3.5. A schematic representation of the elasmobranch model of CO₂ excretion, i.e. CO₂ excretion at the gill of *Squalus suckleyi* [Note that the Pacific spiny dogfish, formerly considered *Squalus acanthias*, has been reclassified as *Squalus suckleyi* (Ebert et al., 2010), but is named *Squalus acanthias* in much of the literature]. Bicarbonate ions (HCO₃⁻) carried in the plasma may enter the RBC for dehydration to CO₂ in the presence of RBC cytosolic CA, or may be dehydrated to CO₂ in the plasma in the presence of pillar cell CA IV. Protons for HCO₃⁻ dehydration are provided by Hb buffering in the RBC and plasma protein buffering in the plasma. Molecular CO₂ diffuses across the gill epithelium down its partial pressure gradient. Some component of this CO₂ likely is hydrated to HCO₃⁻ and H⁺ within the gill epithelial cells, in the presence of branchial cytosolic CA, which may be a different CA isoform than RBC cytosolic CA. Protons and HCO₃⁻ generated within acid- or base-secreting cells of the branchial epithelium are used by ion-transport proteins for purposes of acid-base regulation (see Piermarini and Evans, 2001; Piermarini et al., 2002; Evans et al., 2005; Tresguerres et al., 2006, 2007; Gilmour and Perry, 2009; Tresguerres et al., 2010). Electroneutral exchangers are drawn as open circles, whereas a filled circle indicates an ATPase. AE, band 3 anion exchanger; CA, cytosolic carbonic anhydrase; CA IV, dogfish carbonic anhydrase IV; Hb, haemoglobin; NHE, Na⁺/H⁺ exchanger; P, pendrin-like anion exchanger; RBC, red blood cell.

dehydration via the RBC (reviewed by [Perry and Gilmour, 2002](#)), and DIDS treatment significantly decreased HCO_3^- dehydration by *Squalus suckleyi* RBCs *in vitro* ([Gilmour et al., 1997](#)). Maintenance of CO_2 excretion in anaemic or DIDS-treated *Squalus suckleyi* implies a substantial role for extracellular CA in CO_2 excretion, a conclusion supported by the impairment of CO_2 excretion detected when extracellular CA in anaemic or DIDS-treated *Squalus suckleyi* was inhibited using either benzolamide or polyoxyethylene-aminobenzolamide (F3500), a CA inhibitor that is restricted to the extracellular environment by its large size ([Gilmour et al., 2001](#); [Gilmour and Perry, 2004](#)).

The involvement of branchial CA IV in CO_2 excretion in *Squalus suckleyi* contrasts with the situation in mammals, where pulmonary capillary endothelial CA IV is present but makes a negligible contribution to CO_2 excretion (for review, see [Henry and Swenson, 2000](#); [Swenson, 2000](#)). In mammals, the RBC is a more favorable environment than plasma for HCO_3^- dehydration owing to CA activity and buffer capacity that are, respectively, 100-fold and 10-fold higher than plasma values ([Henry and Swenson, 2000](#); [Swenson, 2000](#)). The higher buffer capacity of the RBC both avoids proton limitations for HCO_3^- dehydration and increases the catalytic efficiency of CA ([Henry and Swenson, 2000](#)), while the presence of endogenous plasma CA inhibitors in some mammals ([Rispen et al., 1985](#); [Hill, 1986](#); [Roush and Fierke, 1992](#)) can accentuate the difference in effective catalytic activity between plasma and RBC by inhibiting endothelial CA IV ([Heming et al., 1993](#)). By contrast, the difference in catalytic activity between plasma and RBC in *Squalus suckleyi* is reduced by the relatively low RBC CA activity (see [Section 3.2.2](#)) and the absence of an endogenous plasma CA inhibitor ([Henry et al., 1997](#)). Similarly, the difference in proton availability between plasma and RBC in *Squalus suckleyi* is reduced by the relatively high buffer capacity of separated plasma (see [Section 3.1](#)), and the absence of a Haldane effect (see [Section 3.1](#)). Owing to the absence of a Haldane effect, HCO_3^- dehydration in the RBC will not benefit from the release of oxylabile protons during hemoglobin oxygenation ([Wood et al., 1994](#); [Perry et al., 1996](#)). Finally, HCO_3^- dehydration that occurs in the plasma bypasses the need for anion exchange, which is the rate-limiting step in RBC-mediated HCO_3^- dehydration in dogfish [*Mustelus canis*, ([Obaid et al., 1979](#)); *Scyliorhinus canicula*, ([Wood et al., 1994](#))]. The collective effect of these factors is to diminish the difference in the relative capacities of the RBC and plasma to contribute to HCO_3^- dehydration at the gill, which allows plasma HCO_3^- dehydration to make a significant contribution to CO_2 excretion in *Squalus suckleyi* and possibly other elasmobranchs.

3.2.4. THE EVOLUTION OF CO₂ EXCRETION PATHWAYS IN VERTEBRATES

A comparison of CO₂ excretion pathways across vertebrates as a whole yields some insight into the evolution of the elasmobranch model of CO₂ excretion, despite very limited data on which to base such speculation (see also [Tufts and Perry, 1998](#); [Tufts et al., 2003](#); [Gilmour and Perry, 2010](#)). Based on extant agnathans, the ancestral vertebrate likely possessed RBCs that lacked the band 3 anion exchanger and contained low levels of CA activity. The RBCs of both hagfish ([Ellory et al., 1987](#); [Peters et al., 2000](#); [Esbaugh et al., 2009](#)) and lamprey ([Nikinmaa and Railo, 1987](#); [Tufts and Boutilier, 1989, 1990](#)) lack functional anion exchange and exhibit levels of CA activity that are low relative to values for teleosts ([Maren et al., 1980](#); [Henry et al., 1993](#); [Esbaugh and Tufts, 2006a](#); [Esbaugh et al., 2009](#)). The potential for the hagfish RBC to contribute to CO₂ excretion is further limited by a small Haldane effect ([Tufts et al., 1998](#)) and the low buffer capacity of its Hb ([Nikinmaa, 1997](#)). However, the presence in the hagfish gill of type IV-like and type XV-like CA activities, coupled with relatively high plasma buffer capacity (the buffer capacity of separated plasma equals that of true plasma), and the absence of an endogenous plasma CA inhibitor provide conditions under which plasma HCO₃⁻ dehydration can make a substantial contribution to CO₂ excretion ([Esbaugh et al., 2009](#)). In agreement with this hypothesis, the majority of the blood total CO₂ load in hagfish is carried in the plasma despite the absence of RBC anion exchange ([Tufts and Perry, 1998](#); [Tufts et al., 1998](#); [Esbaugh et al., 2009](#)). A similar pattern is present in elasmobranchs (or at least *Squalus suckleyi*). Although RBC anion exchange appears in this group, other factors limiting RBC contributions to CO₂ excretion remain, including low CA activity and the absence of a Haldane effect, whereas the presence of branchial CA IV together with high plasma buffer capacity allow for a significant contribution of plasma HCO₃⁻ dehydration, collectively resulting in dual reliance on both plasma and RBC (see [Section 3.2.3](#)). In both hagfish and elasmobranchs then, O₂ transport and CO₂ transport are effectively uncoupled, with O₂ delivery being dependent upon the RBC and CO₂ excretion exhibiting substantial dependence on the plasma.

In the presence of Hb with a strong Bohr-Haldane effect, O₂ and CO₂ transport can be coupled to benefit both O₂ delivery and CO₂ excretion (reviewed by [Brauner and Randall, 1998](#)). Addition of CO₂ to the blood in the tissues lowers Hb-O₂ binding affinity (Bohr effect) to enhance O₂ delivery to the tissues, whereas elimination of CO₂ at the gas exchange organ reverses this effect to the benefit of O₂ loading. As O₂ binds to Hb, it drives off oxylabile protons (Haldane effect) that can then be used for HCO₃⁻ dehydration to benefit CO₂ excretion, whereas deoxygenation of the blood

at the tissues increases the proton-binding capacity of Hb to the benefit of CO₂ loading into the blood. The large Haldane effect of lamprey Hb is critical for effective CO₂ transport by the blood (Tufts and Perry, 1998). Membrane-associated CA activity does not appear to be present in the lamprey gill (Henry et al., 1993), which precludes a role for catalyzed dehydration of HCO₃⁻ in the plasma, and the absence of functional RBC anion exchange (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989, 1990) leaves the RBC as the site of CO₂ transport in the blood (Tufts and Boutilier, 1989). Proton removal via the pronounced Haldane effect and a secondarily active Na⁺/H⁺ exchanger allow CO₂ to be loaded into the blood while HCO₃⁻ ions are retained within the RBC (reviewed by Nikinmaa et al., 1995; Nikinmaa, 1997; Tufts and Perry, 1998). The tight linkage between O₂ and CO₂ transport is retained in teleosts. Again, the gill appears to lack membrane-associated CA activity (Henry et al., 1988, 1993; Henry et al., 1997; Gilmour et al., 1994, 2001, 2002; Stabenau and Heming, 2003; Georgalis et al., 2006), which places reliance for catalyzed HCO₃⁻ dehydration solely on the RBC. Rapid anion exchange at the RBC membrane (Cameron, 1978; Romano and Passow, 1984; Jensen and Brahm, 1995) allows efficient utilization of the plasma for CO₂ carriage, while the benefit of the marked Haldane effect (Jensen, 1991) is maximized by high RBC CA activities (Maren et al., 1980; Henry et al., 1997; Esbaugh et al., 2004) that ensure rapid CO₂ hydration/HCO₃⁻ dehydration reactions with proton production/consumption in close proximity to Hb. It is this pattern of CO₂ excretion that appears to have been largely retained through the tetrapods. Although pulmonary capillary endothelial CA IV is present in tetrapods (Whitney and Briggie, 1982; Zhu and Sly, 1990; Waheed et al., 1992; Stabenau and Heming, 2003), the RBC dominates CO₂ excretion owing to high RBC CA activity and buffer capacity (Henry and Swenson, 2000; Swenson, 2000).

Thus, two basic strategies for CO₂ excretion appear to be present among vertebrates. In one, represented in hagfish and elasmobranchs, the Bohr-Haldane effect is small or absent, O₂ and CO₂ transport are uncoupled, and CO₂ excretion relies to a significant extent on dehydration of HCO₃⁻ in the plasma catalyzed by branchial extracellular CA activity. The second strategy is utilized by lamprey, teleost fish, and tetrapods, and relies on RBC CA to catalyze CO₂ hydration/HCO₃⁻ dehydration reactions; CO₂ excretion is tied to the RBC by coupling it to O₂ uptake. However, with only a handful of species having been studied in any detail, such inferences about patterns of CO₂ excretion and the evolution of CO₂ excretion pathways across vertebrates broadly and fish in particular must remain speculative.

4. CONCLUSIONS AND PERSPECTIVES

Gas transport and exchange have been most thoroughly investigated in just a few, small, sedentary elasmobranch species. Consequently, models of O₂ transport and CO₂ excretion are largely based on information from the so-called “dogfish” sharks, *Scyliorhinus canicula*, *Scyliorhinus stellaris*, *Squalus acanthias*, and *Squalus suckleyi*. Larger and more active elasmobranchs are often dangerous and difficult to use in experiments, and thus data collection from these species has been largely opportunistic. In recent years, researchers have been able to maintain juveniles of large shark species in a laboratory setting, allowing more in depth analysis of the respiratory characteristics of the shortfin mako, *Isurus oxyrinchus*, and sandbar sharks, *Carcharhinus plumbeus* (e.g., Sepulveda et al., 2007; Brill et al., 2008; Wegner et al., 2012). Field physiology (see Bernal and Lowe, 2015) is likely to prove an important tool for future investigations into the respiratory physiology of large elasmobranchs. An excellent example of direct physiological measurements carried out on juveniles and field physiology studies of adults is provided by recent work on the infamous white shark, *Carcharodon carcharias*, a large, regionally-heterothermic shark that does not fare well in aquaria. The O₂ consumption rates of juvenile white sharks were measured (Ezcurra et al., 2012), and a field physiology study was used to estimate routine metabolic rates and feeding requirements of adult white sharks (Semmens et al., 2013), providing great insight into this shark’s ecophysiology.

Generally, elasmobranch Hbs exhibit a high intrinsic affinity for O₂, a high buffering capacity, and weak to moderate cooperativity. There appears to be a division between species in which Hb displays little to no sensitivity to allosteric effectors, and those that exhibit marked Bohr effects and sensitivities to ATP. A high Hb-O₂ affinity causes the O₂ capacitance of the blood ($\Delta\text{CO}_2/\Delta\text{PO}_2$) to be steep and spread over a smaller range at a low *PO*₂. Consequently, elasmobranchs tend to work within a wide portion of the oxygen equilibrium curve, drawing from venous O₂ stores during increased aerobic demand. There are exceptions to this general model (e.g., *Negaprion brevirostris*) and further research into cardiorespiratory adjustments during exercise in elasmobranchs is needed, especially in the high performance lamnid sharks. Elasmobranch RBCs are large, appear to maintain a steady-state volume through exceptional sodium pump activity and RVD mechanisms, and do not possess adrenergic RBC pHi regulation. However, evidence indicates that not all elasmobranchs adhere to this model of RBC function and pH regulation (e.g., *Carcharhinus plumbeus*), clearly warranting research in a wide phylogenetic range of elasmobranchs, as well as in hypoxia- and anoxia-tolerant elasmobranchs.

The blood respiratory properties and Hb-O₂ binding characteristics have been thoroughly investigated for only a few elasmobranchs (e.g., *Squalus acanthias* and *Carcharhinus plumbeus*), but a complete picture of O₂ flux in these species has yet to be composed. A recent resurgence in O₂ equilibria studies has updated standard methods to incorporate modern technology, which in turn has allowed the development of high resolution and high-throughput systems to generate oxygen equilibrium curves on microvolumes of blood (Clark et al., 2008; Lilly et al., 2013; Oellermann et al., 2014). It is hoped that this development will lead to further phylogenetic analyses of Hb function and the elasmobranch Bohr effect. A careful hypothesis-driven investigation of the evolution of the “stingray Bohr effect” in the myliobatid rays is clearly worthy of attention.

As with blood respiratory properties and Hb-O₂ binding characteristics, CO₂ excretion has been thoroughly investigated in only a handful of elasmobranch species, with the most information being available for dogfish. Several lines of evidence suggest that CO₂ excretion in dogfish relies on HCO₃⁻ dehydration in both plasma and RBCs passing through the gill, a strategy that differs from the essentially exclusive reliance on RBCs in other vertebrates, including teleost fish. In dogfish, dehydration of HCO₃⁻ in the plasma is catalyzed by branchial CA IV, with the requisite proton supply being assured by the substantial plasma buffering found in elasmobranch fish. At the same time, the capacity of the RBC to contribute to HCO₃⁻ dehydration is constrained by low RBC CA activity and the absence of a Haldane effect. Clearly there is a pressing need to determine whether this ‘dogfish’ model of CO₂ excretion applies to other elasmobranch species. Molecular and biochemical characterization of branchial and RBC CA isoforms, evaluation of the Haldane effect, and quantification of plasma buffering in a range of elasmobranch fish will be important first steps in determining how likely it is that elasmobranchs beyond dogfish rely on dual plasma and RBC HCO₃⁻ dehydration.

Despite the central roles of O₂ uptake and CO₂ excretion in the success of elasmobranchs, our knowledge of these processes in elasmobranchs as a group has been defined by the thorough investigations carried out in just a few species. The grand challenge ahead lies in achieving a more representative understanding of gas transport and exchange in elasmobranchs as a group.

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ORGANIC OSMOLYTES IN ELASMOBRANCHS

PAUL H. YANCEY

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Marine elasmobranchs are hypoionic osmoconformers. Their extra- and intracellular fluids accumulate organic osmolytes for osmotic balance, mainly urea and methylamines such as trimethylamine N-oxide (TMAO), higher intracellularly at about 2:1 in shallow species, even in embryos and starvation. The relatively few euryhaline species reduce osmolytes only partially, and become hyperosmotic regulators, while permanent freshwater species have almost no organic osmolytes. Urea binds to and unfolds proteins while methylamines promote folding, thermodynamically counteracting each other at 2:1. Both also provide buoyancy. Deep-sea elasmobranchs increase TMAO and reduce urea, possibly because TMAO also

counteracts protein-destabilizing effects of hydrostatic pressure. Recent studies show TMAO coordinates water molecules in a complex excluded from protein backbones (“osmophobicity”). This entropically favors folded protein conformations, and counteracts urea and pressure unfolding effects. Osmolytes are made in the liver or obtained from diets and retained by adaptations of gill, kidney, intestine, rectal gland. Regulatory mechanisms and evolutionary history are incompletely understood.

1. INTRODUCTION

Osmotic balance – especially for maintenance of cell volume – is a fundamental challenge of homeostasis for all organisms. Excessive swelling or shrinking of cells, due to osmosis across semipermeable plasma membranes, changes cellular concentrations (which in turn alters reaction rates, ion interactions with macromolecules, etc.), and may mechanically damage membranes. Cell shrinkage occurs from evaporation or hyperosmotic solutions (external solutions with solute concentrations higher than the cell), while swelling occurs in hypo-osmotic solutions such as freshwater. The potential for swelling damage is worse in animals than in organisms with rigid cell walls that prevent osmotic swelling by turgor pressure.

First, some definitions are in order. The focus of this chapter is osmolytes: small molecules whose concentrations are used to maintain cell volume by balancing intra- and extracellular solute concentrations and are typically regulated in response to osmotic disturbances. Familiar examples of osmolytes are salt ions – Na^+ plus Cl^- – whose concentrations in mammalian plasma are tightly regulated in part to protect cells from swelling and shrinking.

How do osmolytes work? Recall the four colligative properties of solutions – raised boiling point, depressed freezing point, lowered vapor pressure, and osmotic pressure – that depend only on the number of dissolved particles in solution and not on their structure, size, or mass. Most relevant here is osmotic pressure, defined as the hydraulic pressure (in units of atmosphere, torr, mm Hg, or pascal) needed to prevent osmosis (water movement across a semipermeable membrane) from an area of low to an area of high solute concentration. The osmotic effect of a dissolved particle is its osmotic coefficient, exactly 1.0 for an ideal solute. Importantly, a dissociating compound splits into multiple particles in solution. Thus, while glucose has a coefficient of 1, NaCl (Na^+ plus Cl^- in solution) would be expected to have a coefficient of 2 and NaH_2PO_4 a coefficient of 4 (Na^+ , H^+ , H^+ and PO_4^{3-} in solution) (however, these salts are not ideal; as explained in the following).

While plant physiologists often measure turgor osmotic pressures, animal biologists are rarely able to measure pressures across membranes, so characterizations of osmotic (im)balances are typically expressed in terms of two related properties – osmolality or osmolarity, defined as follows.

- Osmolality is directly proportional to osmotic pressure and thus other colligative properties, and to molality – moles of solute per kilogram of solvent (water). Specifically, 1 millimolar (millimoles/kg water) yields 1 milliosmole/kg water, abbreviated mOsm/kg. Osmolality does not change with temperature or hydrostatic pressure and is measurable by laboratory osmometers (which determine freezing point or vapor pressure and calculate osmolality by colligative equivalence).
- Osmolarity is proportional to molarity (M) – moles of solute per liter solution (abbreviated Osm, or mOsm for millimolar). Biologists make chemical solutions using molarity, because that is the relevant factor for chemical reactions; thus, many use mOsm rather than mOsm/kg because of familiar units and because one can quickly estimate osmolarity from molarity. For example, a physiologist finding shark plasma with 400 mM urea estimates the osmolarity contribution at 400 mOsm. However, osmolarity is problematic because: (i) unlike osmolality, it is not a colligative property and cannot be measured; (ii) it changes with temperature and pressure; (iii) the amount of solute dissolved changes the volume of water (for most solutes, a 1 M solution has a higher solute concentration than a 1 molal solution); (iv) not all solutes behave ideally, so estimates of mOsm from M values can be significantly off (Robertson, 1989). A biologically relevant example is NaCl: if ideal, its osmotic coefficient would be 2.0, but in reality it is not fully dissociated in solution, so its coefficient is 1.86 at 25°C at mammalian concentrations (Hamer and Wu, 1972). Thus 150 mM NaCl gives 279 mOsm, not 300 mOsm. Even using osmotic coefficients is not sufficient, as they change in complex solute mixtures. Finally, nondissociating organic osmolytes can also have nonideal coefficients (Section 3).

Thus osmometry that empirically yields osmolality is always preferred over osmolarity. These two terms are often confused and indeed, osmolality is often erroneously called osmolarity in many publications (Erstad, 2003). Therefore, osmolality in mOsm/kg will be used for specific data in this chapter; however, because biologists are used to thinking in molarity, mOsm will be used when discussing general patterns. Molarity will also be used when discussing concentrations relevant to solution interactions.

Before turning to elasmobranchs, it is useful to remember three key osmotic benchmarks – 0 mOsm for pure water, 300 mOsm for basic cell

Table 4.1
Benchmark osmolarities in biology (in mOsm)

| Benchmark | Milieu | Implications for organisms |
|-----------|---|--|
| 0–15 | Freshwater 0 mOsm = pure water | Freshwater organisms and marine animals that migrate into low-salinity (i.e., hypo-osmotic) waters must cope with potential overhydration and cell swelling |
| 300 | Intracellular milieu created by universal solutes of most cells: inorganic ions K^+ , HCO_3^- , $(H)PO_4^{3-}$, etc., metabolites, proteins, nucleic acids, etc. | Many animal groups have internal osmolalities close to this (e.g., mammals including human average 290 mOsm/kg). Levels cannot be reduced greatly due to critical cell molecules |
| 1000 | Seawater at about 34 ppt, a common value in open seas. About 86% due to Na^+ plus Cl^- | Marine organisms in this potentially hyperosmotic habitat must have adaptations to prevent dehydration and cell shrinkage |

solutes, and 1000 mOsm for seawater. As described in [Table 4.1](#), these are the approximate values for the most common internal and habitat osmolarities.

2. OSMOCONFORMERS VERSUS OSMOREGULATORS

In marine animals, two broad strategies have evolved to deal with the osmotic challenges of a potentially dehydrating environment: (i) osmoconformation – having an internal osmolality similar to the environment; and (ii) osmoregulation – maintaining an internal osmolality significantly different than the environment. The term “osmoregulation” is often used broadly (covering all osmotic systems), but here it will be used in this stricter definition, akin to thermoconformation versus thermoregulation (body temperature similar to, vs. regulated to be different than, the environment, respectively). Elasmobranchs exhibit both osmotic strategies, depending on the environment ([Fig. 4.1](#)).

2.1. Osmoconforming: Elasmobranchs in the Oceans

Although basic cell constituents yield only about 300 mOsm ([Table 4.1](#)), most marine animals osmoconform to their environment; that is, they are isosmotic. The advantage is that there is little tendency to gain or lose water. To achieve conformity, extracellular fluids (ECFs) have high NaCl (readily available from seawater); while in contrast, cellular osmolality is elevated

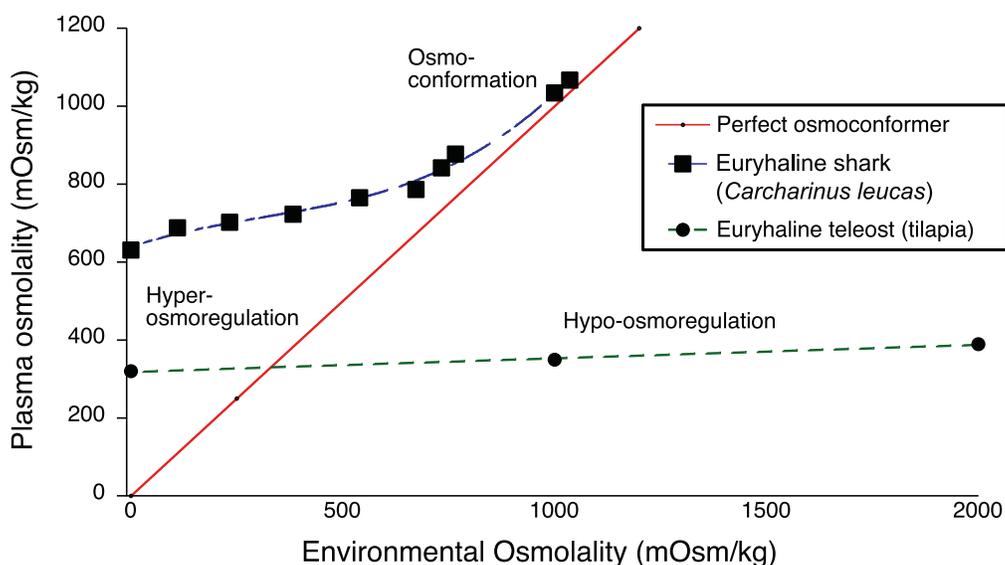


Figure 4.1. Overview of fish plasma versus environmental osmolalities. See text for details; 0 is pure freshwater; 1000 is average seawater. Data for tilapia plotted from [Fiess et al. \(2007\)](#), for bull shark *Carcharinus leucas* plotted from [Pillans and Franklin \(2004\)](#).

predominately with certain low-molecular-weight solutes called organic osmolytes. Thus, cells might have 300 mOsm of basic cell solutes, slightly higher inorganic ion levels, and 600 mOsm of organic osmolytes. Euryhaline species regulate cellular organic osmolytes more than inorganic ions for cell volume maintenance. As reviewed elsewhere ([Yancey et al., 1982](#); [Yancey and Siebenaller, 2015](#)), organic osmolytes fall into four broad categories across the spectrum of life ([Fig. 4.2](#)): small carbohydrates; amino acids; methylamine and methylsulfonium solutes; and urea. The selective “rationales” for these types, as well as for combinations in elasmobranchs, will be discussed in Section 3.

2.1.1. OVERVIEW OF OSMOCONFORMERS

To put elasmobranchs in context, it is helpful to start with other conformers. In most marine invertebrates, cells use mainly free - and -amino acids ([Fig. 4.2](#)) and methylamines as organic osmolytes. However, ECFs resemble seawater with Na^+ and Cl as the major osmolytes. Water is balanced due to osmoconformation (e.g., 1000 mOsm inside and outside). Hagfish (Myxini) are one of the few vertebrate groups that osmoconform, with osmolytes in ECF and cells similar to invertebrates ([Fig. 4.3](#)). Other vertebrates that osmoconform are Chondrichthyes, considered next; the coelacanth ([Fig. 4.3](#)); a few polar (not discussed further) and hadal teleost fishes ([Section 3.4](#)); and the crab-eating frog *Rana cancrivora*, which uses mainly urea ([Gordon et al., 1961](#)).

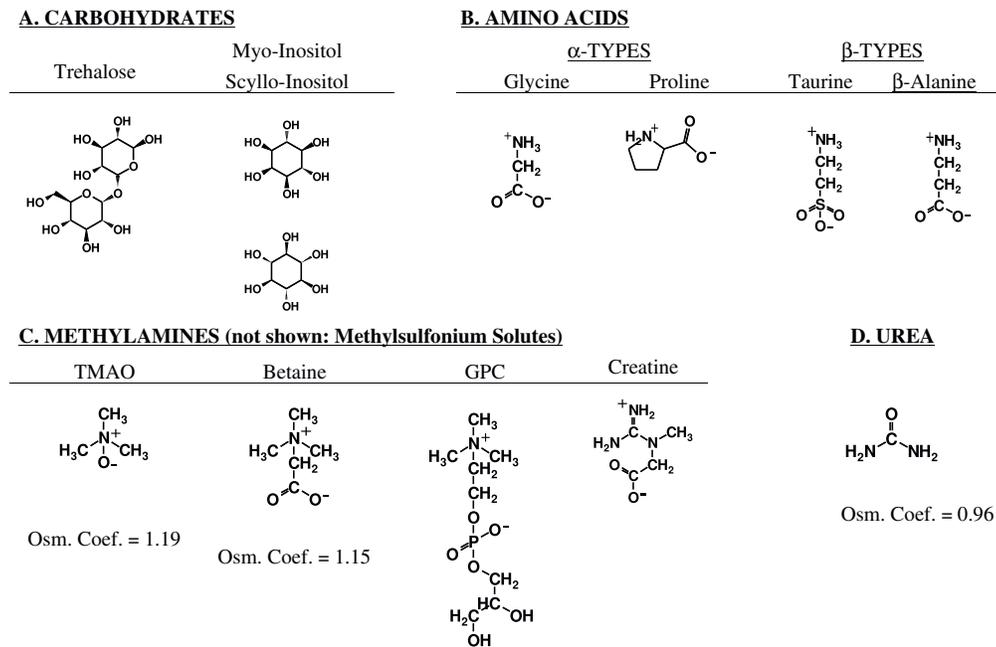


Figure 4.2. Structures of typical organics osmolytes found in marine organisms. TMAO, N-trimethylamine oxide; GPC, glycerophosphorylcholine; Osm. Coef., osmotic coefficient (1.0 for ideal solute).

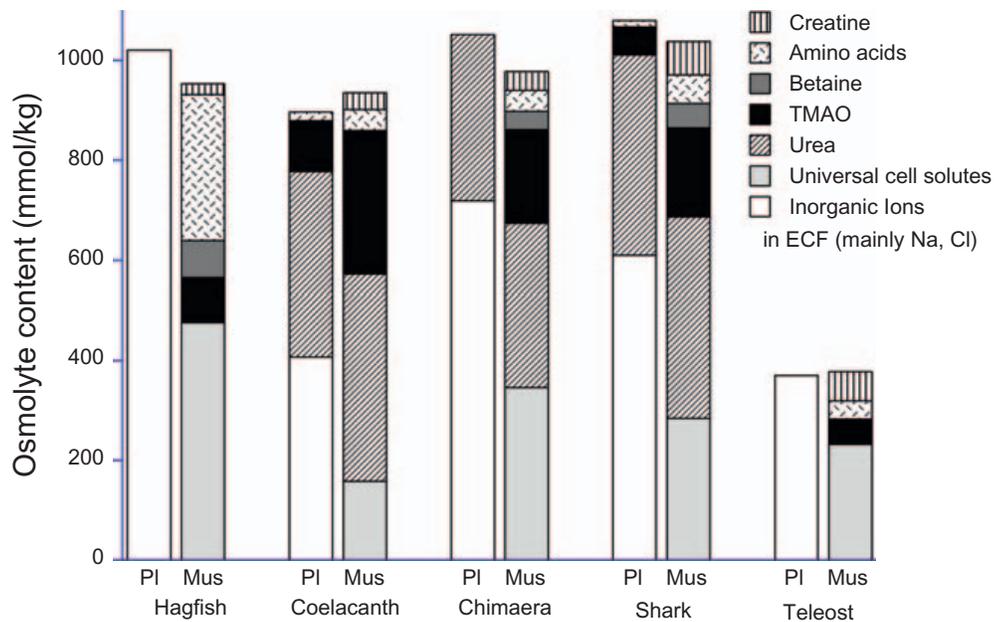


Figure 4.3. Osmolyte contents of plasma (PI) in mM and in muscles (Mus) estimated as mmol/kg cell water for marine fishes. Data for hagfish *Myxine glutinosa* and *Chimaera monstrosa* from Robertson (1976); coelacanth from Lutz and Roberston (1971); shark *Scyliorhinus canicula* from Robertson, 1989; and teleost *Pleuronectes flesus* from Lange and Fugelli (1965). Modified from Yancey (2001).

2.1.2. ELASMOBRANCHS: UREOSMOTIC WITH 2:1 UREA:METHYLAMINES

Marine Chondrichthyes (elasmobranchs and holocephalans) are classified as osmoconformers (Fig. 4.1), but differ from invertebrate/hagfish patterns in several ways. First, they are ionoregulators with a mixture of organic and inorganic osmolytes in their ECFs. As a result, their ECFs (plasmas) have considerably lower concentrations of inorganic osmolytes (especially Na^+ plus Cl) than the environment (Fig. 4.3, Chimaera and Shark, Pl bars). This may reflect evolutionary origins (Section 5).

Second, marine elasmobranch species are sometimes but not always reported to be slightly hyperosmotic (e.g., 1050 mOsm/kg internally vs. 1000 mOsm/kg seawater; reviewed by Ballantyne and Fraser, 2013). Hyperosmolality would create a slow osmotic flux of water inwards, reducing the need to drink seawater (in contrast to marine teleosts; see Section 2.2). The lack of consistency in reporting of hyper- versus isosmotic states may be due to failure to measure seawater osmolality at exact site of capture (Ballantyne and Fraser 2013), species differences, different methodologies, or lack of rapid regulation in an individual moving in a salinity gradient.

Third, elasmobranchs' primary organic osmolytes are (Figs. 4.2–4.4; Table 4.2):

- Urea: typically at 300–500 mM in ECF and cells alike, in shallow marine species. Urea is not found in marine invertebrates, and, as discussed later, is unusual in other ways. First detected in elasmobranchs by Staedeler and Frerichs (1859), it was not recognized as an osmolyte until the early 1900s. Homer Smith (1936) coined the term “ureosmotic” for chondrichthyan osmotic physiology. They are also “ureotelic” – having urea as primary nitrogenous waste.
- Trimethylamine N-oxide (TMAO) and other methylamines (betaine or N-trimethylglycine or glycine betaine; sarcosine or N-methylglycine; and creatine or N-carbamimidoyl-N-methylglycine); and possibly homarine or N-methylpicolinic acid (Dove et al., 2012): totaling about 40–90 mM in ECF and 150–250 mM in many tissues, at least in most shallow marine species. TMAO was first discovered in sharks in 1909 (reviewed by Smith, 1936); it is usually the most concentrated methylamine. Creatine, due to its role in phosphate energy storage, is typically high only in muscle tissue.
- Various polyols such as myo- and scyllo-inositol, and free -amino acids including taurine and -alanine (Table 4.2; Fig. 4.4A); these vary considerably among species and tissues (Sherman et al., 1978; King and Goldstein, 1983; Steele et al., 2005; Laxson et al., 2011) but are usually lower in concentration than the methylamines and urea.

In the 1970s an important pattern was recognized (Yancey and Somero, 1978): the ratio of urea to TMAO is widely found at approximately 2:1

Table 4.2

Osmolyte concentrations in plasma (mM) and contents in tissues (mmol/kg tissue) of selected elasmobranchs. Blanks are solutes not measured. Abbreviations: Bet, betaine; Sarc, sarcosine; Creat, creatine; FAA, free amino acids (mainly taurine, β -alanine); U:T, U:M, ratio of urea to TMAO or to summed methylamines. Note that values come from homogenizing whole tissues, so ratios inside cells are somewhat lower; for example, for a muscle having 30% ECF/plasma with 70 mM TMAO, and 70% intracellular fluid with 200 mM TMAO, the homogenate will have ~ 161 mM $[(200 * 0.7)+(70 * 0.3)]$.

| Species | Habitat | Tissue | Na | K | Cl | Urea | TMAO | Bet | Sarc | Creat | FAAs | U:T | U:M |
|--|---------------|--------|-----|-----|-----|------|------|-----|------|-------|------|------|------|
| <i>Aptychotrema vincentiana</i> | Benthic | plasma | 264 | 7 | 287 | 337 | 71 | | | | | | |
| S. Shovelnose ray | | muscle | 34 | 98 | 29 | 357 | 162 | 57 | 11 | | | 2.20 | 1.55 |
| <i>Furgaleus ventralis</i> | Benthopelagic | plasma | 260 | 7 | 238 | 342 | 70 | | | | | | |
| Whiskery shark | | muscle | 39 | 98 | 32 | 357 | 166 | 57 | <1 | | | 2.15 | 1.60 |
| <i>Heterodontus portusjacksoni</i> | Benthopelagic | plasma | 317 | 7 | 306 | 353 | 81 | | | | | | |
| Port Jackson shark | | muscle | 119 | 69 | 105 | 364 | 161 | 45 | <1 | | | 2.26 | 1.77 |
| <i>Carcharhinus obscurus</i> | Pelagic | plasma | 279 | 14 | 275 | 346 | 75 | | | | | | |
| Dusky shark | | muscle | 41 | 106 | 43 | 365 | 160 | 16 | 69 | | | 2.28 | 1.49 |
| (<i>Leuco-</i>) <i>Raja erinacea</i> | Benthic | plasma | 299 | 5 | | 361 | 39 | | | | | | |
| Little skate | | muscle | | | | 417 | 49 | 40 | 32 | 40 | 55 | 8.51 | 2.6 |

Data from [Withers et al. \(1994b\)](#) except for little skate (plasma from [Forster and Goldstein, 1976](#); muscle from [Steele et al., 2005](#)).

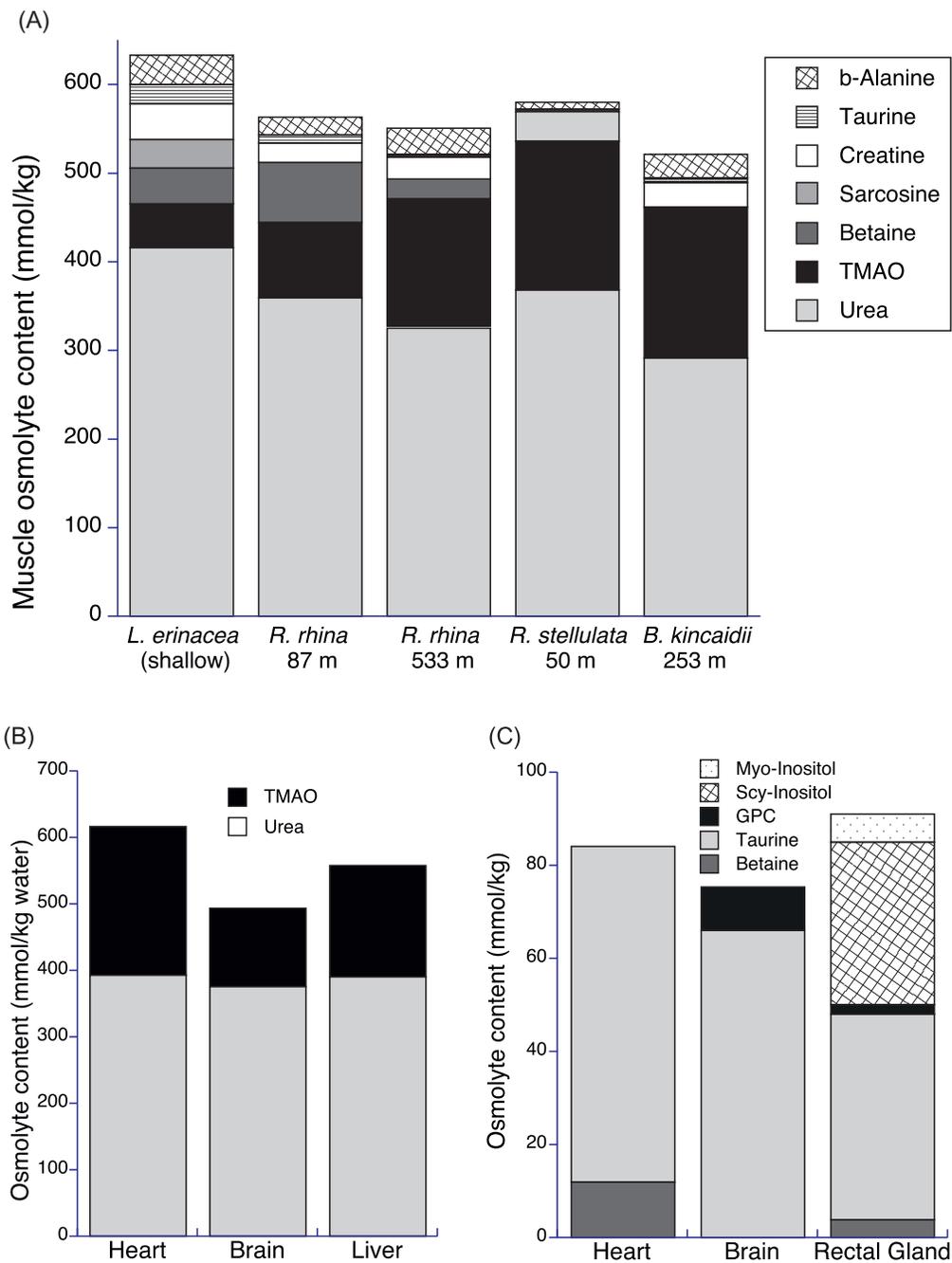


Figure 4.4. Organic osmolyte contents (mmol/kg) in elasmobranch tissues. (A) Skate muscle osmolytes as mmol/kg tissue mass. Depths of capture are shown below species names. Species and sources: (*Leuco-*)*Raja erinacea*: Steele et al., 2005; *Raja rhina*, *Raja stellulata*, *Bathyraja kincaidii*: Laxson et al., 2011. (B) Urea and TMAO contents per tissue water in various organs of the shark *Mustelus manazo* (plotted from Suyama and Tokuhiro, 1954). (C) Non-urea, non-TMAO osmolytes in organs of *Squalus acanthias*, per tissue water. Scy-Inositol, scyllo-inositol. Modified from Yancey (2001).

intracellularly, at least in shallow stenohaline marine elasmobranchs, and closer to 1.5:1 with other methylamines included (Table 4.2). Note that values such as those in Table 4.2 do not give true ratios because urea is equal intra- and extracellularly while the other osmolytes are higher intracellularly. Since tissue values are derived by homogenizing whole tissues, urea will not be diluted during the process, but intracellular osmolytes will be diluted by the ECF. See Table 4.2 for details. The significance of urea:methylamine ratios will be discussed in detail in Section 3.2.

Although TMAO is generally the dominant methylamine in marine species, in tissues of many brackish and euryhaline Batoidea (rays including skates), a higher ratio (e.g., 2.6, Table 4.2) is found for urea:methylamines (sarcosine, betaine, TMAO) plus -amino acids (taurine and -alanine; Table 4.2, Fig. 4.4A) (King and Goldstein, 1983; Steele et al., 2005; Treberg et al., 2006). Also in most species, methylamines (especially TMAO) are highest in cardiac and skeletal muscle while other tissues tend to have somewhat less (Fig. 4.4A,B), with erythrocytes (cells with the most “intimate” contact with plasma) having urea and TMAO concentrations similar to those of plasma (Sulikowski et al., 2003). In such nonmuscle tissues, other osmolytes including taurine, betaine, GPC (Fig. 4.2), and inositols are elevated at the expense of TMAO (Fig. 4.4C).

2.2. Osmoregulating: Elasmobranchs at Low Salinities

Again, marine elasmobranchs, although ionoregulators, are not osmoregulators in the sense used here. The situation is different at lower salinities, however, as shown in Fig. 4.1 for a euryhaline shark. This shift to osmoregulation will be discussed after a brief overview of vertebrate osmoregulation in general.

2.2.1. OVERVIEW OF OSMOREGULATION

Most marine vertebrate groups not discussed earlier are osmoregulators: they actively maintain a homeostatic (but not constant or “homostatic”) internal milieu hypo-osmotic to the surrounding (hyperosmotic) seawater in part by using ATP to pump out excess ions. For example, most teleost fishes (Actinopterygii, Teleostei) in shallow, nonpolar seas maintain 350–400 mOsm/kg internally regardless of the environmental osmolality (Figs. 4.1 and 4.3). Teleosts must drink seawater to make up for osmotic water losses, and their gills must transport out excess NaCl. In freshwater, osmotic gradients are reversed so teleosts are hyperosmotic (Fig. 4.1), with gills transporting salts inwards and kidneys removing excess water. Cells are thus protected from swelling or shrinking by the regulated ECF of the whole animal, and organic osmolytes have been largely disregarded.

However we now know the situation is more complex. Consider the highly euryhaline Mozambique tilapia (*Oreochromis mossambicus*): its plasma increases from 320 to 390 mOsm/kg when acclimated from 0 to 2000 mOsm/kg (Fig. 4.1). To balance this modest increase in plasma osmolality, due mostly to NaCl, cells accumulated organic osmolytes – myo-inositol, glycine and taurine – though at much lower levels than in osmoconformers (Fiess et al., 2007).

We also know that certain organs within osmoregulators, perhaps most notably the mammalian kidney medulla, have osmoconforming cells using organic osmolytes. See Section 3.

Not only teleosts but virtually all animals in low-salinity and freshwater must be hyperosmotic osmoregulators to some extent because they cannot reduce their internal osmolalities below a certain level, especially not to 0 mOsm! This includes elasmobranchs.

2.2.2. OSMOREGULATING ELASMOBRANCHS

Of the roughly 1100 known elasmobranch species, only about 170 have been considered to be capable of living in lower salinity habitats and of those, only perhaps 50 or so can live long-term in freshwater (Martin, 2005). This is a much lower percentage of freshwater species than is found among teleost fishes (40%). These 170 elasmobranchs can be grouped into the following osmotic categories (adapted from Martin, 2005; Ballantyne and Fraser, 2013):

- **Marginal/Brackish Species:** the largest group, these are coastal species that can tolerate reduced salinities, but not freshwater. At lower salinities they become regulators, with inorganic and organic osmolytes reduced but becoming very hyperosmotic. A well-studied example is the little skate, (*Leuco-*)*Raja erinacea*, of northwestern Atlantic coastlines. As shown in Table 4.3, when acclimated to 50% seawater, it reduces plasma mOsm by only 25%, due to modest reductions in Na⁺ (and presumably Cl), urea, and TMAO. Marginal species are considered to be poor osmoregulators; for example, the dogfish *Squalus suckleyi*, placed into 60% seawater, developed cataracts and gained weight from osmosis due to poor regulation of Na⁺, urea, and water balance (Guffey and Goss, 2014).
- **Euryhaline Migrating Species:** this group consists of only 13 species of dasyatid and pristid rays and carcharhinid sharks. They are found in the oceans and far up rivers. The best-studied species are the bull shark *Carcharinus leucas*, found worldwide in warm seas and well up many rivers (e.g., into Lake Nicaragua and 4000 km up the Amazon), and the Atlantic Ray *Dasyatis sabina* of the western Atlantic and Gulf of Mexico. These stay healthy while becoming osmoregulators (hyperosmotic) at low salinities, but reducing their osmolytes more than do the marginal/brackish species (Fig. 4.1, Table 4.3).

Table 4.3
Plasma concentrations (mM) and osmolalities (mOsm/kg) of selected elasmobranchs
in different salinities

| Osmotic category | Salinity (mOsm/ kg) | Na ⁺ | K ⁺ | Cl ⁻ | Urea | TMAO | Plasma osmolality |
|---|------------------------|-----------------|----------------|-----------------|------|------|----------------------|
| Marginal/Brackish | | | | | | | |
| <i>(Leuco-)Raja erinacea</i> ^a | SW | 299 | 5 | | 361 | 39 | 965 |
| Little Skate | 50% SW | 217 | 4 | | 264 | 30 | 719 |
| <i>Leucoraja ocellata</i> ^b | SW (922) | 246 | 4 | 232 | 398 | 31 | 930 |
| Winter Skate | 50% (455) | 151 | 3 | 150 | 243 | 22 | 556 |
| Euryhaline migrating | | | | | | | |
| <i>Dasyatis sabina</i> ^c | SW | 310 | 7 | 300 | 394 | | 1034 |
| Atlantic Stingray | FW | 212 | 5 | 208 | 196 | | 621 |
| <i>Carcharhinus leucas</i> ^d | SW | 304 | 6 | 315 | 293 | 47 | 940 |
| Bull Shark | FW | 221 | 4 | 220 | 151 | 19 | 595 |
| Euryhaline FW | | | | | | | |
| <i>Himantura signifer</i> ^e | FW | 167 | | 164 | 44 | 0 | 416 |
| FW Whipray | 60% SW | 231 | | 220 | 83 | 0 | 571 |
| Stenohaline FW | | | | | | | |
| <i>Potamotrygon aiereba</i> ^f | FW | 156 | 6 | 178 | 4.2 | 0 | 305 |
| Aiereba Stingray | | | | | | | |

Abbreviations: SW, seawater; FW, freshwater. Blanks indicate solutes not measured

^aForster and Goldstein (1976)

^bSulikowski et al. (2003)

^cPiermarini and Evans (1998)

^dPillans et al. (2004, 2005)

^eTam et al. (2003)

^fDuncan et al. (2009)

- Permanent Freshwater Euryhaline Species: this group consists of a few species of Southeast Asian and African dasyatid rays that live and breed in freshwater but can be acclimated to higher salinity. They are thought to have only recently (in geological terms) been trapped in freshwater, perhaps explaining their osmotic state: they too retain some urea despite it being detrimental for osmotic balance (Ballantyne and Fraser, 2013). The whiptail ray *Himantura signifer* is an example (Table 4.3). Though it lives in freshwater, it can be acclimated up to 600 mOsm/kg brackish water (Tam et al., 2003). No TMAO is found in these fish; see Section 3.2.2.
- Permanent Freshwater Stenohaline Species: this group consists of 30 species of South American stingrays in the family Potamotrygonidae, which have been in freshwater much longer than the previous group. In contrast to other groups, these retain almost no TMAO or urea (their kidneys having lost the ability for urea reabsorption; Section 4); moreover, they only have low levels of enzymes for urea synthesis and cannot

accumulate it if placed in higher-salinity water (Griffith et al., 1973; Anderson, 1991; Tam et al., 2003). Their internal osmolalities are near the archetypal 300 mOsm/kg (Table 4.3). They may have lost ancestral ureosmotic abilities after a long period of evolutionary adaptation (Gerst and Thorson, 1977). Interestingly, at least one species, *P. motoro*, placed in 400 mOsm brackish water becomes an osmoconformer in part by increasing (like an invertebrate) amino-acid osmolytes (glutamate, glutamine and glycine) but not urea intracellularly (Ip et al., 2009).

3. PROPERTIES OF ORGANIC OSMOLYTES

One of the first questions that arose after the discoveries of organic osmolytes is this: why do cells use organic solutes, which may cost energy and/or tie up compounds that have other uses, instead of “free” inorganic ions (especially NaCl) to raise cellular osmolality when needed?

3.1. Inorganic Ions Versus Compatible Solutes

Immediately following an osmotic disturbance, uptake and release of inorganic osmolytes, especially K^+ , Na^+ , and Cl, are regulated by many cell types to adjust cell volume, but usually only over a small range of osmolalities (Hoffman et al., 2009). Cells capable of surviving prolonged osmotic changes regulate organic osmolytes more than inorganic ones for volume regulation (Yancey and Siebenaller, 2015). For example, in little skates (*Leuco-*)*Raja erinacea* transferred to lower salinity, ECF inorganic and organic osmolytes are reduced equally, but organic osmolytes decrease two-fold more than K^+ and other ions (King and Goldstein, 1983). In part this use of organic over inorganic osmolytes may be to minimize ion effects on membrane potentials. However, a major reason is related to solute effects on macromolecules.

3.1.1. INORGANIC IONS AS PERTURBANTS

Recall that the major inorganic ions in most cells are K^+ , bicarbonate and phosphates, not Na^+ or Cl (Table 4.1). NaCl concentrations above normal are often quite disruptive of macromolecular functions (e.g., enzymatic reactions, RNA synthesis; Yancey et al., 1982). Moreover, while K^+ salts at levels found inside cells do not inhibit and can even stimulate macromolecular functions, they cause perturbation at higher concentrations. An example is shown in Fig. 4.5 for a non-elasmobranch (i.e., teleost) protein.

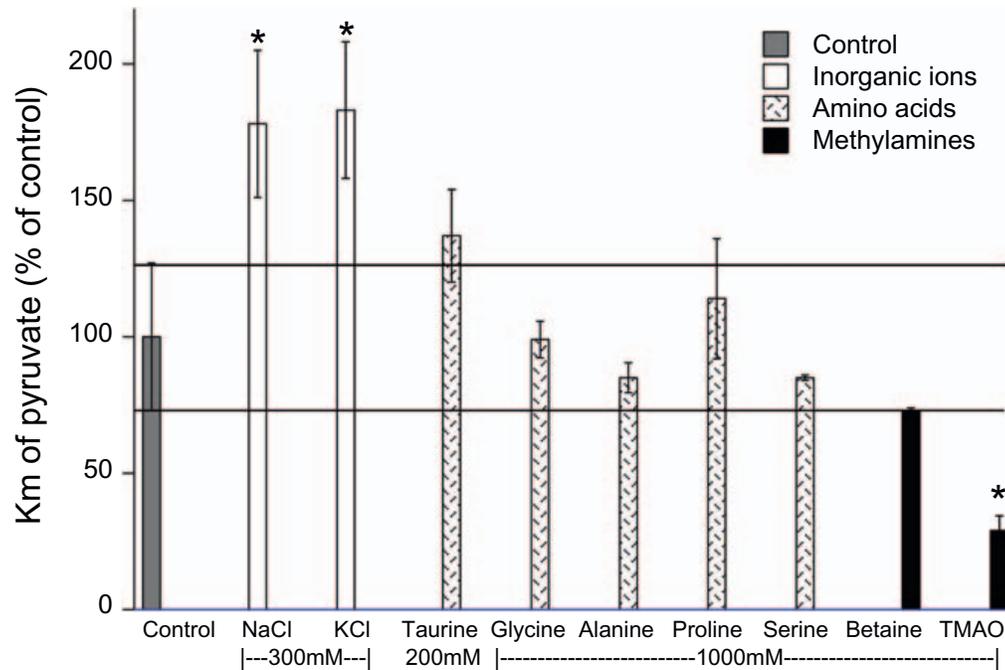


Figure 4.5. Osmolyte effects on a model enzyme, lactate dehydrogenase (LDH). Data are for Michaelis–Menten constant, K_m , of pyruvate for bluefin tuna LDH, plotted from [Bowlus and Somero \(1979\)](#). Horizontal bars show 95% confidence limits for control value; * indicates significantly inhibited from control. As a higher K_m indicates weaker binding, this shows that inorganic osmolytes are inhibitory, neutral amino acids are compatible even at high concentrations, and methylamines (especially TMAO) enhance binding.

Exposing living cells to external NaCl (but not urea) concentrations higher than normal (or to any hyperosmotic condition using impermeant solutes) is also deleterious, at least initially. Problems typically correlate with elevated intracellular ions ([Burg et al., 2007](#)). Several studies have found up-regulation of chaperoning stress proteins, indicating that proteins are partly denatured during cell shrinkage; for example, in salmon cells (which suffer reduced protein synthesis; [Smith et al., 1999](#)), and in mammalian cells (which also suffer inhibited translation and transcription, even apoptosis; [Burg et al., 2007](#)).

High extracellular NaCl (at 500–1000 mOsm/kg), due to elevated intracellular ions, also induces nonrepaired double-stranded breaks in DNA in living cells from terrestrial nematodes, mammals, and even marine flatworms, nemertean, annelids, molluscs, and crustacean, which are well adapted to 1000 mOsm/kg ([Kültz and Chakravarty, 2001](#); [Dmitrieva et al., 2006](#)). Nematode and mammalian cells that do not compensate with organic osmolytes undergo apoptosis following this salt-induced DNA breakage ([Burg et al., 2007](#)).

3.1.2. ORGANIC OSMOLYTES AS COMPATIBLE SOLUTES

The initial explanation for the use of organic rather than inorganic osmolytes inside cells was the “compatibility” hypothesis of [Brown and Simpson \(1972\)](#), later extended by other groups ([Clark and Zounes, 1977](#); [Wyn Jones et al., 1977](#); [Bowlus and Somero, 1979](#)). The hypothesis, based on studies with proteins *in vitro*, states that (in contrast to salts) most organic osmolytes do not disrupt macromolecular function, even at high concentrations. The perturbing effects of ions and absence of negative effects of compatible osmolytes are generally similar with proteins from species with or without high levels of organic osmolytes. This led to an important corollary of the hypothesis: effects of salts and organic osmolytes should be general features of protein-solute-water interactions, not of specific adaptations in protein structure. Use of compatible organic osmolytes should not create significant disruptions over a wide range of external salinities, because their concentrations can be varied with little effect on proteins. The use of taurine and other neutral amino acids as osmolytes in many marine animals (including elasmobranchs) has often been explained by this hypothesis. See example in [Fig. 4.5](#).

Mammalian (renal) and marine invertebrate cells that use organic osmolytes thrive even with unrepaired DNA breaks. Recently, osmotically adaptable mammalian renal cells have been found to restrict the DNA breaks to “gene deserts” – chromosomal regions with no genes. This may explain why osmolyte-using adaptable cells thrive under osmotic stress despite persistent breaks ([Dmitrieva et al., 2011](#)). However, neither the mechanism for restricting breaks to these chromosomal areas nor a direct causal connection with organic osmolytes is known. Nor have elasmobranch cells been examined for this. Perhaps by having ECF salts lower than seawater (using urea which does not cause DNA breaks; see next section), elasmobranchs did not have to adapt to this problem. However, we do not know whether urea retention or lowered NaCl evolved first or simultaneously.

3.2. Urea Versus Methylamines: Counteracting Solutes

The situation for urea and TMAO seems to be different. In short, neither is compatible in the original sense. However, when studied in combination, they revealed a new principle of osmolyte functions, as will be discussed.

3.2.1. UREA: BENEFICIAL AND PERTURBING EFFECTS

To a physiologist, urea appears to be an excellent osmolyte for use in intra- and extracellular fluids. [Ballantyne and Fraser \(2013\)](#) noted that

the ideal solute to replace inorganic ions...should be uncharged, small, and rapidly diffusing...Urea has all of these properties...[which] would be beneficial in situations of changing external salinity...

Urea indeed equilibrates readily across most membranes (depending on lipid composition; [Poznansky et al., 1976](#)), resulting in concentrations about equal in cells and ECFs ([Fig. 4.3](#)). Note that rapid equilibration requires facilitated urea transporters (UTs; see below). As a small uncharged (albeit polar) molecule, urea can diffuse slowly via simple or “basal” diffusion through lipid membranes (on the order of 10^7 cm/sec for common membranes; [Goodman, 2002](#)). Elasmobranch erythrocytes, for example, may rely on this nonspecific process ([Carlson and Goldstein, 1997](#)). However, for faster movement, up to 10^3 cm/sec, UTs are required to quickly balance extra- and intracellular osmolalities during osmotic changes ([Walsh et al., 1994](#); [McDonald et al., 2006](#)). Rapid equilibration via UTs may explain why urea osmotic shock does not cause DNA breaks in mammalian renal cells ([Burg et al., 2007](#)). Urea may also rapidly equilibrate among cell compartments; for example, *R. erinacea* mitochondria have an ancient bidirectional urea carrier ([Rodela et al., 2008](#)). In contrast, charged osmolytes are much higher inside cells than outside due in part to very low basal membrane permeability, only moving across membranes via transporters (slow compared to UTs; [Goldstein and Kleinzeller, 1987](#)).

However, these possible benefits of urea beg two major questions. First, why are most other organic osmolytes larger than urea and often charged, thus impermeant to membranes? The answer may lie in the evolutionary origins – see [Section 5](#). Second, if urea facilitates osmotic adjustments, why are there so few euryhaline elasmobranchs? One answer might be that many so-called stenohaline/marginal marine species are in fact more euryhaline than thought ([Hazon et al., 2003](#)). Recently, a supposedly marginal species, the South American skate *Zapteryx brevirostris*, was found to acclimate quite well down to 100 mOsm environmental water ([Wosnick and Freire, 2013](#)). The other possible answer has to do with urea’s negative properties.

From a biochemist’s viewpoint, urea seems an odd evolutionary selection for a major osmolyte because, at the concentrations found in marine chondrichthyans and mammalian kidneys, urea it is not “compatible”: it disrupts many macromolecular structures and functions.

Importantly, these negative effects are also seen with many (though not all) elasmobranch proteins; see [Table 4.4A](#) and [Section 3.2.2](#). These fishes clearly survive indefinitely with high internal concentrations of urea. How is this achieved? There are three different hypotheses, each with supporting evidence.

Table 4.4
Urea and methylamine effects on biological systems

| A. Examples of negative effects of urea on biological systems. | |
|---|--|
| System tested and effect of urea | Reference |
| Teleost gill respiration inhibited 25–37% in 300 mM urea | Yancey (1985) |
| Mammalian collagen assembly inhibited by urea >100 mM | Fessler and Tandberg (1975) |
| Microtubules of protozoa and mammals inhibited by urea >100 mM | Shigenaka et al. (1971) and Sackett (1997) |
| Rabbit heart contraction inhibited 40% in 300 mM urea | Schmidt et al. (1972) |
| Frog embryos: 50% mortality of in 110 mM urea | McMillan and Battle (1954) |
| Mammalian renal cells <i>in vitro</i> : 60–80% decreased growth rate in 200 mM urea | Yancey and Burg (1990) |
| Hemoglobin (Hb) of shark <i>Squalus acanthias</i> : 400 mM urea raises O ₂ affinity, reduces sensitivity to ATP | Weber (1983) |
| B. Examples of urea-insensitive systems in elasmobranchs | |
| Elasmobranch system tested with urea | Reference |
| Glycine-cleavage enzyme complex of skate mitochondria: kinetics unaffected; unlike inhibited teleost and clam homologous complexes | Moyes and Moon (1990) |
| Elasmobranch cartilage: highly sulfated, urea-resistant | Mathews (1967) |
| Hemoglobins from sharks, skates, rays: oxygen binding unaffected; unlike amphibian and mammalian hemoglobins | Bonaventura et al. (1974) |
| Membranes of elasmobranch liver mitochondria: much higher percentage of saturated fatty acids than in other fishes, for urea resistance? | Glemet and Ballantyne (1996) |
| Shark immunoglobulin IgNAR | Feige et al. (2014) |
| C. Examples of urea-requiring systems in elasmobranchs | |
| Elasmobranch protein tested with urea alone | Reference |
| Shark lens protein: improper structure without at least 250 mM urea | Zigman et al. (1965) |
| Muscle lactate dehydrogenases (LDHs): with no urea, pyruvate K _m values lower for elasmobranch homologues than for other vertebrates; raised by 400 mM urea to normal vertebrate range | Yancey and Somero (1978) |
| D. Examples of urea enhancement with TMAO inhibition | |
| Protein or process tested with urea and/or TMAO | Reference |
| Shark muscle lactate dehydrogenases (LDHs): catalytic rate and active-site flexibility | Yancey and Somero (1980) and Zhadin and Callender (2011) |
| Skate mitochondrial respiration | Ballantyne and Moon (1986) |

(Continued)

Table 4.4 (Continued)

| D. Examples of urea enhancement with TMAO inhibition | |
|---|------------------------|
| Protein or process tested with urea and/or TMAO | Reference |
| Dogfish shark 5'-monodeiodinase: substrate binding (however, TMAO activates catalytic rate) | Leary et al. (1999) |
| Rabbit actomyosin: sliding motor activity and ATPase rate inhibited by TMAO (which, however, enhances stability in counteraction to urea) | Kumemoto et al. (2012) |

1. Urea Resistance. First, some proteins appear to be insensitive or resistant to urea's effects. Examples are listed in [Table 4.4B](#). Elasmobranch immunoglobins are particularly interesting because they occur extracellularly where there is insufficient counteraction ([Section 3.2.2](#) below).
2. Urea Requirement. There are at least a few elasmobranch proteins that appear to need urea for proper function; see examples in [Table 4.4C](#). Having at least some proteins with a urea requirement might explain why most elasmobranchs in low-salinity waters do not eliminate urea fully even when it is no longer needed for osmotic balance ([Ballantyne and Fraser, 2013](#)). Perhaps only Potamotrygonid rays have evolved long enough to eliminate the need for urea ([Table 4.3](#)).
It has long been known that shark hearts *in vitro* require high urea concentration in the perfusing fluids to function properly ([Simpson and Ogden, 1932](#); [Wang et al., 1999](#)). However, because the heart cells probably contained impermeable TMAO, while the external urea equilibrated across the membrane, it could be that the heart required a proper urea:TMAO ratio intracellularly. This leads to the final mechanism.
3. Counteracting Osmolytes. The destabilizing effects of urea may be counteracted by osmolytes that stabilize proteins. This may be the primary adaptation and is discussed extensively next.

3.2.2. METHYLAMINES: BENEFICIAL AND PERTURBING STABILIZERS

Urea, as a protein destabilizer, is clearly not a compatible solute. Many studies, often unrelated to osmolyte research, have shown that other osmolyte-type solutes, especially methylamines, are also not compatible in the original sense. Rather, they promote folding of polypeptides into native states and enhance assembly of multimeric proteins. Importantly, by doing so, stabilizing solutes can offset many effects of various destabilizing agents

(Yancey et al., 1982). For example, it has been known for decades that most natural osmolytes can increase protein thermostability *in vitro* (offsetting heat denaturation), although for most, nonphysiologically high concentrations are required. One of the first physiological examples of stabilization discovered was that between urea and TMAO (and other methylamines) in elasmobranchs (Yancey and Somero, 1979, 1980). Those studies found opposite effects of urea and methylamines on elasmobranch protein structural stability and enzyme kinetics. Moreover, the effects were additive such that they canceled each other, most effectively at about a 2:1 urea:TMAO ratio (Fig. 4.6A,C), similar to elasmobranch cellular levels, roughly 400:200 mM (Tables 4.1–4.3, Fig. 4.3). This phenomenon was termed “counteracting osmolytes” (Yancey and Somero, 1979, 1980). Like compatibility, counteraction occurs whether a protein is from a urea-accumulating organism or not—for example, the first studies included mammalian as well as elasmobranch proteins, and numerous subsequent studies continue to demonstrate urea–methylamine counteraction with proteins from numerous taxa including mammals (Fig. 4.6B) and bacteria, as well as with more complex systems (e.g., shark muscle fibers, Altringham et al., 1982). Counteraction between urea and TMAO has also been found on fluidity of elasmobranch erythrocyte membranes, although opposing solute effects may be exerted through integral membrane proteins rather than on membrane lipids (Barton et al., 1999).

As noted earlier, permanent freshwater elasmobranchs do not appear to have TMAO even in the Euryhaline Freshwater group that retains some urea (Table 4.3). However, the urea levels in these species (<100 mM) are not high enough to perturb most proteins (Yancey, 2005), so counteraction may not be required.

Urea-methylamine counteraction is not unique to elasmobranchs. The osmoconforming coelacanth has urea and TMAO at about 2:1 (Fig. 4.3). Methylamines also co-occur with urea in the mammalian renal medulla, a finding inspired by the elasmobranch discovery (Bagnasco et al., 1986). Cells in the medulla of the mammalian kidney, which can have a high urea content due to the urinary concentrating mechanism, have close to a 2:1 ratio of urea to the methylamines glycerophosphorylcholine (GPC) and betaine (Fig. 4.2), with GPC regulated in parallel to urea (Peterson et al., 1992). GPC and betaine can counteract urea’s effects on renal proteins (Burg et al., 1996).

The stabilizing ability of organic osmolytes by themselves led to the term “chemical chaperone,” as a counterpart to “molecular chaperones” term for heat-shock and similar proteins (Welch and Brown, 1996). That protein conformation is key to many counteracting effects is revealed in studies that examine both chaperone types. For example, in acute heat shock

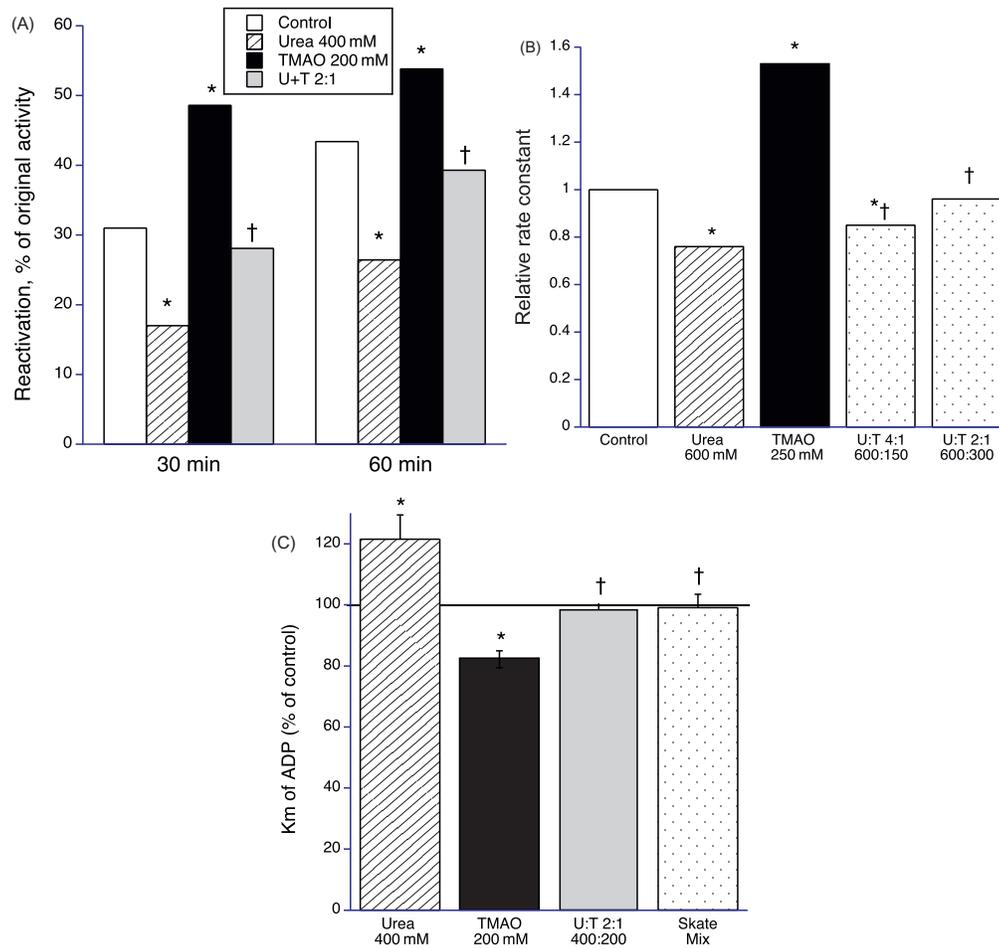


Figure 4.6. Stabilizing and counteracting effects of TMAO and other organic osmolytes against urea. *Significantly different from control; †significant counteraction. (A) Original discovery of TMAO’s chaperoning ability: refolding in a physiological buffer of white shark A_4 -lactate dehydrogenase after acid denaturation, without (control) and with urea and/or TMAO as indicated. Data plotted from Yancey and Somero (1979). (B) Recent example of similar phenomenon: polymerization of mammalian actin, plotted as relative rate with and without the solutes indicated and showing optimal counteraction at 2:1 urea:TMAO (U:T). Horizontal bar at 1.0 is control level. Data plotted from Hatori et al. (2014). (C) ADP K_m values for muscle pyruvate kinase (PK) from round stingray (*Urolophus haleri*) with urea and/or TMAO, and/or “Skate mix” (400 mM urea, 65 mM TMAO, 55 mM sarcosine, 30 mM betaine, and 50 mM β -alanine, the osmolyte composition of one skate muscle; King and Goldstein, 1983). Horizontal bar at 100 is control level. Data plotted from Yancey and Somero (1980).

(Fig. 4.7A), a physiological TMAO concentration reduced hsp70 accumulation 7-fold in isolated dogfish erythrocytes (Kolhatkar et al., 2014) and 2-fold in isolated dogfish choroid plexus (Villalobos and Renfro, 2007). Since Hsp70 expression is induced by protein denaturation, this and related studies indicate that methylamines reduce protein denaturation.

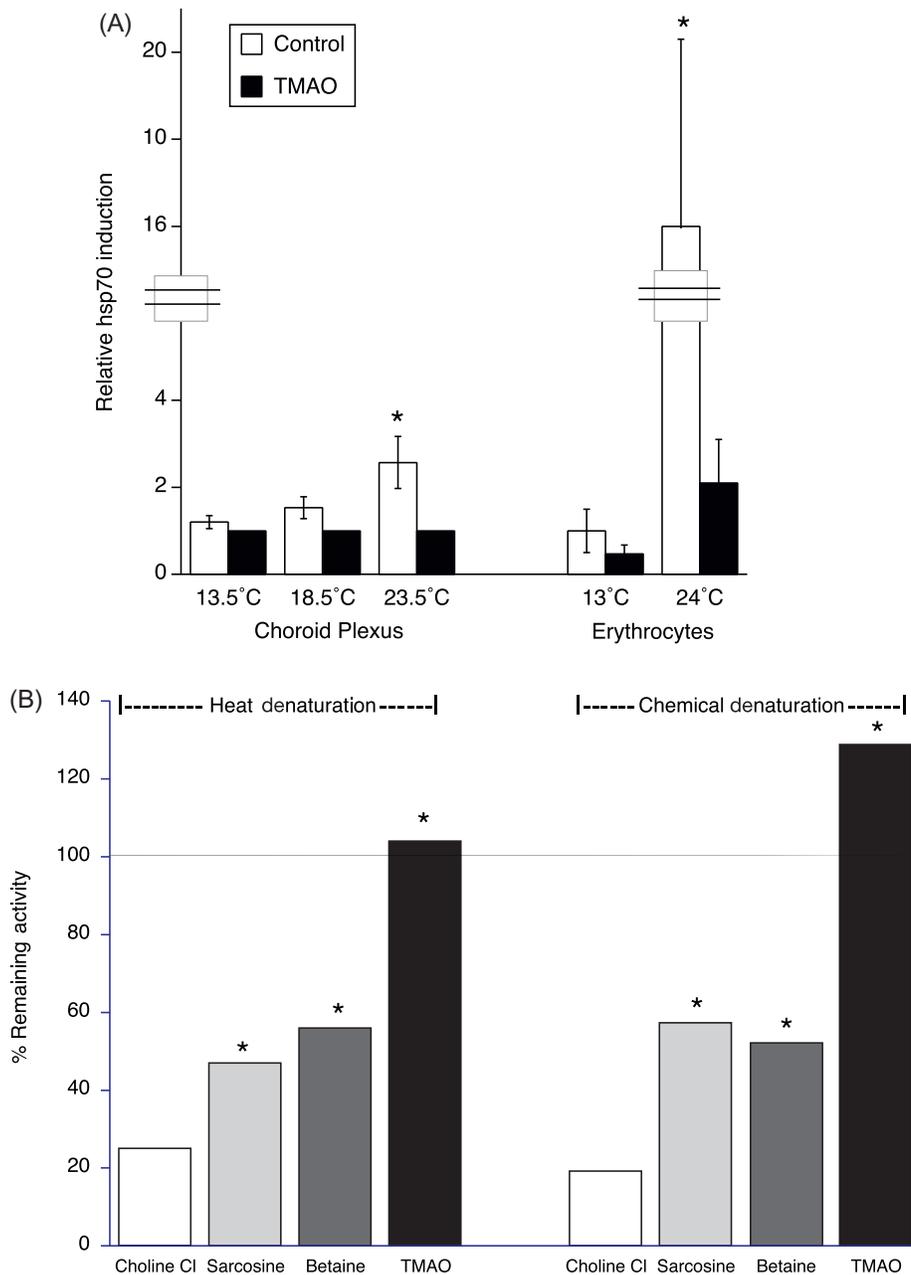


Figure 4.7. Stabilizing and counteracting effects of TMAO and methylamines against thermal and chemical stresses. (A) Hsp70 expression (relative to actin) in shark (*Squalus acanthias*) lateral choroid plexus with acute heat shock (normal temperature 13–13.5°C) without and with 72 mM TMAO, and in dogfish erythrocytes without and with 63 mM TMAO. Results show lower induction with TMAO present. Indicates significant difference. For choroid plexus, hsp70 induction with TMAO present was set as the comparison level and normalized to 1.0 for each temperature. For erythrocytes, induction for control at 13°C was set at 1.0. Plotted for plexus from Villalobos and Renfro (2007) and for erythrocytes from Kolhatkar et al. (2014). (B) Trypsin denaturation. Left: trypsin was heated for 10 min at 60°C without or with 3.5 M of the

Importantly, TMAO is usually a stronger stabilizer than other osmolytes including betaine, while trimethylamines in turn are usually stronger than di- and mono-methylamines like sarcosine. Fig. 4.7B illustrates this for thermal and chemical stresses on a model protein, trypsin (see also Sackett, 1997; Yancey, 2005). TMAO's superb stabilizing abilities may explain why TMAO is the dominant non-urea osmolyte in most ureosmotic marine fishes. Moreover, some non-methylamine osmolytes are more "classically" compatible; for example, glycine – common in marine invertebrates but not urea-rich cells – can stabilize many proteins against temperature but shows no ability to counteract effects of urea (Khan et al., 2013) or pressure (Section 3.4) on proteins. The widespread taxon-independent effects must involve universal protein-water-solute mechanisms (Section 3.5).

Recall that in many brackish and euryhaline rays, but not in stenohaline marine elasmobranchs, a roughly 2:1 ratio is only reached with urea if all methylamines and –amino acids are included (Table 4.2, Fig. 4.4A). Betaine, sarcosine and –alanine can also counteract urea (Yancey and Somero, 1979, 1980) (see also Section 3.5.4), although not quite as effectively as TMAO, at least individually (as noted earlier). However in a mixture there may be synergism (Fig. 4.6C; also Section 3.5.4). Also, the non-TMAO osmolytes may be "cheaper" to make or easier to get dietarily than TMAO (see Section 4) in low salinities where prey have no TMAO and elasmobranchs presumably use more energy for osmoregulation (Treberg et al., 2006). Importantly, *Raja rhina* in shallow water (Fig. 4.4A, 87 m) has a complex mixture, but in deep water it shifts to TMAO dominance (Fig. 4.4A, 533 m); see Section 3.4.

3.2.3. THE YIN AND YANG OF UREA VERSUS METHYLAMINES

It is important to recognize an often-overlooked aspect of the counteracting hypothesis: the term "counteracting" applies to both classes of solutes. That is, neither urea nor methylamines by themselves are necessarily neutral or beneficial; that is, too much TMAO relative to a destabilizer might be detrimental due to over-stabilization, such as proteins too rigid to flex properly and forming nonfunctional aggregates (reviewed by Yancey, 2005). Indeed, occasionally urea and TMAO have been found to have "reversed" effects; examples are listed in Table 4.4D. Moreover, there may be nonadditive effects; for instance, for cultured mammalian renal cells, high

methylamines indicated, then given substrate for activity measurements. Right: trypsin was exposed to 30% trifluoroethanol without or with 3.5 M of the methylamines indicated, then given substrate for activity measurements. Activity was zero in both cases in the absence of methylamines. Horizontal bar at 100 is original control level prior to denaturation. Data plotted from Levy-Sakin et al. (2014).

urea or betaine alone added to the culture medium greatly reduced cell growth. However, adding both partly or fully restored normal growth (Yancey and Burg, 1992).

Studies on starved elasmobranchs are quite revealing. Unfed winter skates (*L. ocellata*) maintained urea/TMAO levels for 45 days (Treberg and Driedzic, 2006) and unfed dogfish sharks similarly maintained the osmolytes for 2 months (Kajimura et al., 2008; Wood et al., 2010). It seems the 2:1 ratio is paramount independently of net osmolality.

Thermostabilization ability can also show detrimental effects. In the shark study on TMAO versus hsp70 of Fig. 4.7A, the heat-stressed tissue suffered more inhibition of transport in the presence of TMAO (with low hsp70 induction) than in its absence (high hsp70 induction) (Villalobos and Renfro, 2007). Trehalose (Fig. 4.2), another strong stabilizer, also reveals perturbing features. When tested with shark ornithine transcarbamolase, the sugar protected the enzyme from thermal denaturation but at the same time reduced its catalytic rate, presumably due to reduced protein flexibility (Bellocco et al., 2005). Similarly, high trehalose concentrations, induced by temperature stress in yeast, protect enzymes at high temperatures, but inhibit them at normal temperatures. This was memorably called the “the yin and yang of trehalose” (Singer and Lindquist, 1998), a phrase that applies well to urea and methylamines.

Negative effects of TMAO in the absence of a destabilizer may explain recent findings that elevated TMAO in human blood is associated with cardiovascular disease (Tang et al., 2013). Perhaps TMAO in some unknown manner enhances atherosclerotic plaques (although TMAO may be correlative, not causative).

3.2.4. EXCEPTIONS

Urea and TMAO do not exhibit counteraction in every situation (Yancey et al., 1982; Mashino and Fridovich, 1987). For example, calcium binding by purified stingray muscle parvalbumin II (an protein that facilitates muscle relaxation by binding calcium) was increased *in vitro* by all osmolytes including urea (Heffron and Moerland, 2008). Moreover, TMAO does not always offset urea inhibition. Yancey et al. (1982) suggested that some urea effects that may not be counteracted, include competitive inhibition of substrate binding, in which urea binds to the active site. Such proteins including LDH in ureosmotic fishes may have evolved a urea requirement, as noted earlier (Yancey and Somero, 1978). Or, they may receive urea protection by other effects *in vivo*; for example, thornback-ray phosphofructokinase is inactivated *in vitro* by low urea but not protected by TMAO; however it is much less sensitive to urea when complexed with actin filaments (Hand and Somero, 1982). Similarly, shark myosin ATPase

activity is just as sensitive to urea as is carp myosin, but this sensitivity is eliminated by actin binding (Kano et al., 1999). Another urea-inhibited elasmobranch protein not protected by TMAO *in vitro* is the dogfish hemoglobin in Table 4.4B. Weber (1983) speculates that lack of TMAO effects evolved to prevent overstabilization, that is, to allow conformational changes necessary for the unusually broad Bohr (pH) effect in this species. Notably, erythrocytes typically have much less TMAO than other cells types (described earlier).

Finally, recall that plasma proteins are exposed to high urea but low methylamines. Recently, Feige et al. (2014) analyzed a shark immunoglobulin (Ig; Table 4.4A) and found 1 more salt-bridge and larger hydrophobic core compared to a homologous human Ig, which explained the shark protein's greater resistance to urea destabilization.

3.3. Salts Versus Methylamines: Counteracting Solutes

Methylamines can sometimes offset perturbing salt effects. This was discovered independently with marine invertebrate enzymes in TMAO by Clark and Zoues (1977), and with plant enzymes in betaine and sarcosine by Pollard and Wyn Jones (1979). TMAO also shows salt counteraction in some mammalian systems (e.g., Desmond and Siebenaller, 2006). Whether salt-methylamine counteraction occurs in elasmobranchs is not known.

3.4. TMAO Versus Pressure: A Piezolyte in the Deep Sea?

The latest discovery of counteraction involves the deep sea, where high hydrostatic pressure can destabilize protein structures and inhibit functions. This occurs whenever there is a positive volume change during a reaction. To cope with this, many deep-sea bacterial, teleost, and invertebrate proteins have evolved structural (intrinsic) changes in amino-acid sequences that reduce volume changes (in poorly understood ways) and so reduce pressure inhibition (Siebenaller and Somero, 1989; Wakai et al., 2014). However, many proteins from deep-sea teleosts and invertebrates are not fully resistant to pressure effects (reviewed by Yancey and Siebenaller, 2015). Indeed, they may also require “piezolytes” (Martin et al., 2002): osmolyte-type solutes that counteract pressure effects.

3.4.1. TMAO IN DEEP-SEA TELEOSTS

The idea of piezolytes began with Gillett et al. (1997) who reported that TMAO contents in teleost muscles increase linearly with depth in teleosts caught from 0 to 2900 m. Also, their ECFs had higher [NaCl] and thus osmolalities above the 350–400 mOsm/kg typically of shallow species. Since

pressure is the only environmental factor that increases linearly with depth, a pressure-counteraction role for TMAO was postulated (see [Section 3.4.2](#)).

Later studies confirmed and extended the pattern across numerous families and species ([Fig. 4.8A](#)): muscle TMAO contents increase both inter- and intraspecifically with depth from 40 mmol/kg tissue to 386 mmol/kg in a hadal species from 7000 m (see [Fig. 4.8A](#) for references). Increasing TMAO elevates muscle osmolalities above the “classic” 300–400 mOsm/kg; indeed muscle of the 7000-m hadal species (the Kermadec Trench snailfish) was almost isosmotic at 991 mOsm/kg ([Yancey et al., 2014](#)).

3.4.2. PIEZOLYTE PROPERTIES OF TMAO

As hypothesized, TMAO at *in situ* concentrations has been found to partly or fully counteract hydrostatic-pressure inhibition of many deep-sea teleost proteins; that is, TMAO appears to be a piezolyte. Moreover, counteracting effects of TMAO versus pressure have been found to be universal, working for a variety of proteins from shallow and terrestrial animals, for growth of yeast under pressure ([Gillett et al., 1997](#); [Yancey and Siebenaller, 1999](#); [Yancey et al., 2001, 2002, 2004](#)), and for a bacterial membrane channel ([Petrov et al., 2012](#)). Examples are shown in [Fig. 4.9](#). Importantly, for one model protein (a teleost LDH), TMAO was found to be the best pressure counteractant ([Fig. 4.9B](#)). The structure of another model protein, staphylococcal nuclease (SNase), was studied by X-ray scattering TMAO by [Krywka et al. \(2008\)](#). They reported: “A drastic stabilization is observed for the osmolyte TMAO, which exhibits not only a significant stabilization against temperature-induced unfolding, but also a particularly strong stabilization of the protein against pressure.”

Other hypotheses for high TMAO including buoyancy are less well supported in terms of being unique to the deep sea, but may also be involved ([Samerotte et al., 2007](#)). For example, [Seibel and Walsh \(2002\)](#) proposed that TMAO is a byproduct of higher lipid production in the deep sea and in elasmobranchs in general. See [Section 5](#).

3.4.3. DEEP-SEA ELASMOBRANCHS AS TMAO-OSMOTIC CONFORMERS

What about marine osmoconformers, especially elasmobranchs, which in shallow waters have much higher TMAO levels than teleosts? There appear to be no studies on elasmobranch proteins under pressure, but their osmolytes show an intriguing pattern. [Kelly and Yancey \(1999\)](#) reported that TMAO increased with depth in some crustaceans, squid, and – of most relevance here – *Bathyraja* skates (2 species). Because all these animals are osmoconformers, an increase in TMAO must be offset with a decrease in other osmolytes. In skates, it was urea that declined as TMAO increased. Muscle urea and methylamine contents in shallow species ranged between

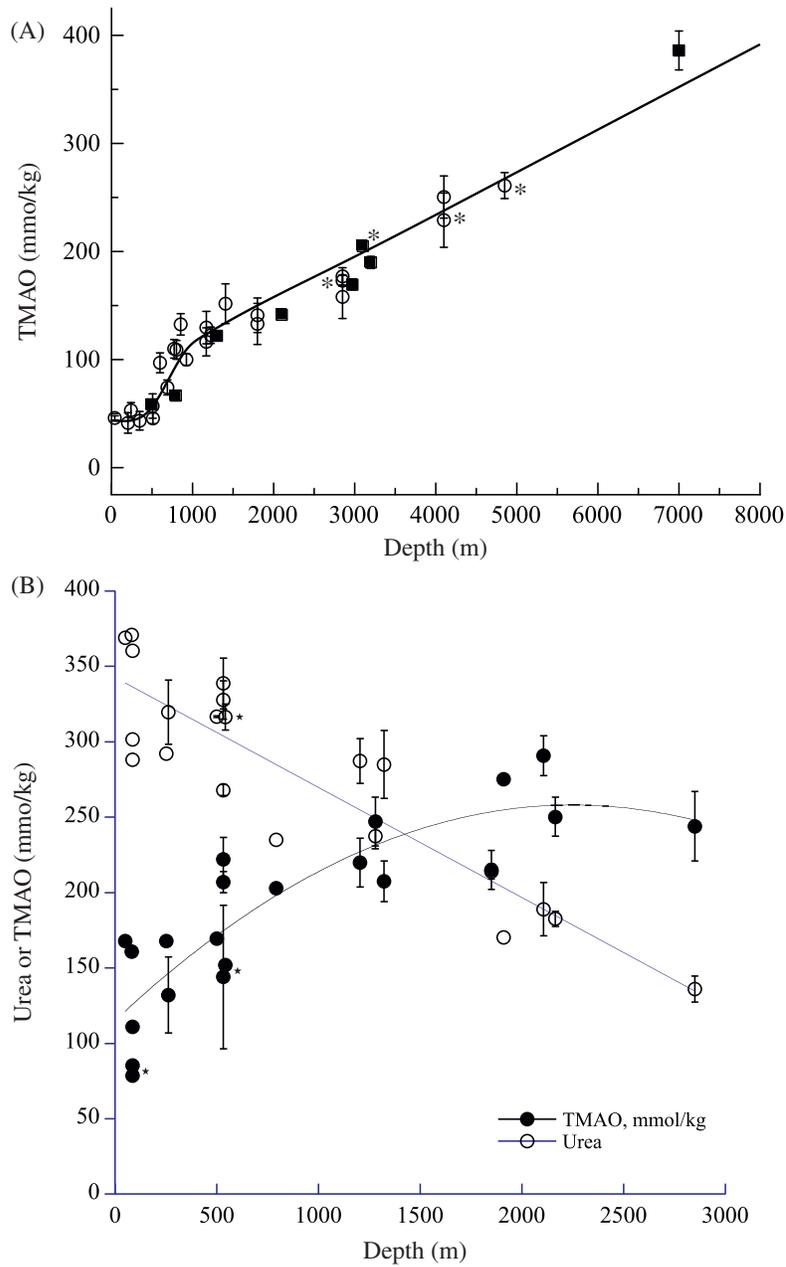


Figure 4.8. Muscle osmolyte contents (mmol/kg) as a function of depth. (A) TMAO in 19 species, 9 families of teleosts. *indicates same species, *Coryphaenoides armatus*. Circles, from Gillett et al. (1997), Kelly and Yancey (1999), Yancey et al. (2004), and Samerotte et al. (2007); squares, from Yancey et al. (2014). Curve fits: lower line for 1997–2007 data, upper line includes 2014 data. Figure used with permission from Yancey et al. (2014). (B) Urea and TMAO in 16 species of elasmobranchs (sharks, skates and other rays) and holocephalans; *Indicates same species, *Raja rhina*. Reprinted with permission from Laxson et al. (2011).

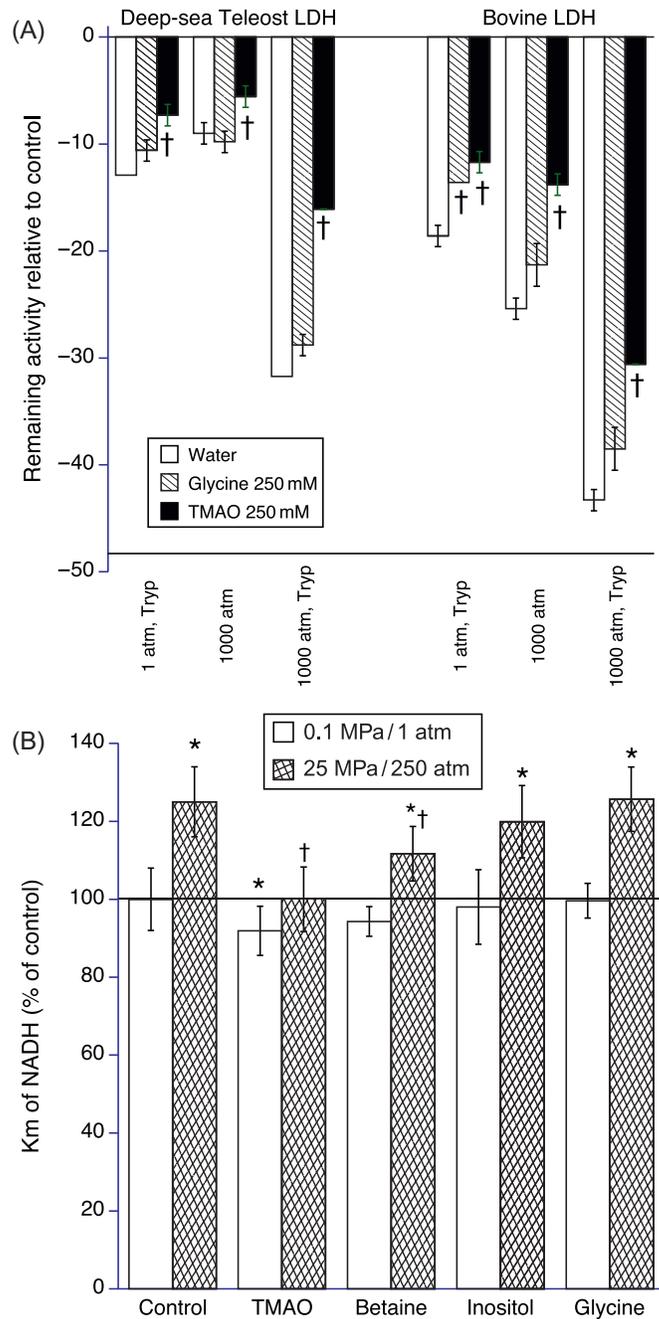


Figure 4.9. Effects of osmolytes on teleost and mammalian enzymes at high hydrostatic pressure. No elasmobranch proteins have been tested. Horizontal bars represent control levels. (A) Loss of activity of muscle lactate dehydrogenases (LDHs) incubated under pressure for a deep-sea teleost *Sebastes altivelis*, and cow *Bos taurus* (data plotted from Yancey and Siebenaller, 1999). Enzymes were incubated with and without 250 mM TMAO or glycine, and without or with trypsin. With trypsin the enzyme suffers greater pressure loss, indicating sub-denaturing changes in conformation. All pressure and trypsin treatments caused significant

80–180 mmol/kg and 300–400 mmol/kg, respectively (near the 2:1 ratio), while a species from 2850 m had TMAO and urea values of 244 mmol/kg and 136 mmol/kg, respectively, yielding a “reversed” ratio of nearly 1:2, and so better termed “TMAO-osmotic” rather than ureosmotic. The concomitant decline in urea with rising TMAO was proposed to maintain osmoconformation, increase TMAO-pressure counteraction, while reducing urea’s destabilizing effects adding to hydrostatic pressure effects (Kelly and Yancey, 1999).

Subsequently, Treberg and Driedzic (2002) reported a deep-sea shark from about 1500 m having about 1:1 urea:TMAO. The pattern was extended for a number of other shark and skate species from numerous depths, shown in Fig. 4.8B. The deep species’ methylamines were dominated by TMAO (Laxson et al., 2011), in contrast to complex mixtures in some shallow skates; note the differences in TMAO and urea for *Raja rhina* caught in shallow and deeper waters (Fig. 4.4A and Fig. 4.8B, *).

Although TMAO may simultaneously serve as an osmolyte and piezolyte in marine animals, this is not always the case. Indeed, amphipods in Lake Baikal, the world’s deepest (freshwater) lake (1642 m), revealed depth-correlated increases (down to 1200 m) in TMAO in muscles (Zerbst-Boroffka et al., 2005). Since osmolytes are detrimental to water balance in freshwater animals, TMAO must have another role. This strongly supports the hypothesis that in marine teleosts and elasmobranchs, increasing TMAO with depth is selected in response to pressure.

3.4.4. PIEZOLYTES AND DEPTH LIMITS

TMAO as a piezolyte may explain two curious depth distributions. The oceans descend to 11,000 m in some trenches, where several invertebrate groups are found, but teleosts have been documented only to 8400 m, apparently absent from the greatest depths (Jamieson and Yancey, 2012; Priede and Froese, 2013). Recently, TMAO was proposed to be the limiting factor, because (as TMAO increases with depth) teleosts become isosmotic at about 8200 m, and greater depths would require more TMAO, higher internal osmolalities, and consequently a complete reversal in osmoregulatory systems (Yancey et al., 2014).

declines in activity relative to non-treated controls (not indicated), but TMAO consistently counteracted pressure († indicates significant protection) while glycine rarely did. (B) NADH K_m of A₄-lactate dehydrogenase (LDH) from a deep-sea teleost (*Coryphaenoides leptolepis*; ghostly grenadier) from 2900 m. Horizontal bar represents control levels. Various osmolytes (indicated below the bars; Inositol is *myo*- type) were all tested at 250 mM, with TMAO having the greatest counteracting effect (* indicates significantly different from 1 atm control; † indicates counteraction against pressure). Data plotted from Yancey et al. (2004).

What about elasmobranchs and holocephalans? [Priede et al. \(2006\)](#) and [Priede and Froese \(2013\)](#) thoroughly documented that Chondrichthyes species and abundances decline precipitously below 3000 m, with few species between 3000–4156 m, and seemingly none deeper than that. The authors hypothesized this absence of chondrichthyans in the abyss is due to their high metabolic needs, in part for the maintenance of enlarged lipid-rich livers.

As an alternative, [Laxson et al. \(2011\)](#) hypothesized an osmolyte limit based on the pattern in [Fig. 4.8B](#). First, the need for osmolytes in elasmobranchs coupled with the oligotrophic nature of the deep sea might result in the inability of these fish to accumulate high enough levels of TMAO to counteract both urea and hydrostatic pressure. The TMAO data in [Fig. 4.8B](#) hint at such a limit, but more species from greater depths are needed to test this. Second, it may be difficult for these fishes to reduce their urea content beyond a certain level. Recall that euryhaline migratory stingrays and sharks moving into freshwater keep urea at levels well above osmoconformation levels ([Table 4.3](#)). Although [Fig. 4.8B](#) show no sign of a urea plateau at depth, no truly deep species (>2500 m) have been captured to analyze. If there is an inability to reduce urea below a certain level, it might be due to structural adaptations in the gill and kidney, which have evolved to retain urea (see [Section 4](#)). Or it might be due to urea-requiring proteins. Finally, urea retention may have remained in deep-sea species (as in euryhaline species) so that urea can be readily increased in the event of vertical migration. Potamotrygonids have almost no urea ([Table 4.3](#)), but then they cannot re-invade estuaries. Finally, of course, these osmolytes may have nothing to do with chondrichthyan depth limits.

3.4.5. COUNTERACTING COMPRESSION OF WATER

A different hypothesis for piezolyte function is based on methylamines' water-expanding effects, as evidenced in their partial molal volumes (PMV, the portion of the volume of a solution due to the molar content of one of the components). TMAO's PMV, for example, is +72.7 cm³/mol, that is, it expands water upon dissolving ([Withers et al., 1994a,b](#)). This might come into play in the deepest oceans where pressure compresses water volume up to 5%. The presence of TMAO in cells could reduce this compression and thus protect cell volume (G.N. Somero, personal communication).

3.5. Physicochemical Mechanisms of (De)stabilization

Because the compatible, (de)stabilizing, and counteracting properties of organic osmolytes are often universal, they are thought to involve broad water-solute-macromolecule interactions, as originally proposed by Clark,

Somero, Yancey and colleagues (Clark and Zounes, 1977; Yancey et al., 1982) and Wyn Jones et al. (1977).

3.5.1. IONS AND UREA AS DESTABILIZERS: PREFERENTIAL INTERACTION

The hypotheses for protein destabilizers fall into two categories: direct binding and indirect through alteration of water structure. For urea, there is some evidence for both (Bennion and Daggett, 2004), but most evidence favors the dominance of direct binding, specifically to the peptide intra-backbone hydrogen bonds crucial to protein stability (Auton et al., 2007; Lim et al., 2009; Hua et al., 2008) and to some amino-acid side groups (Chanchi and Garcia, 2011). Salt ions bind to charged amino acids in proteins. Binding leads to the phenomenon termed “preferential interaction” (Timasheff, 2002), which will lead to unfolding of macromolecules because that maximizes the favorable surface interactions (Fig. 4.10A, right).

In terms of indirect effects, urea binds to water less well than water does to itself, as suggested by its osmotic coefficient being slightly less than 1 (Fig. 4.2D). However, effects on water are weak and there is no consistent correlation between urea’s effects on water structure on its denaturing abilities (Pezos and Gai, 2012).

3.5.2. METHYLAMINES AS STABILIZERS: PREFERENTIAL EXCLUSION AND OSMOPHOBICITY

The discovery of TMAO-urea counteraction has spawned, and continues to stimulate, numerous studies in many laboratories on possible mechanisms. In contrast to urea, organic stabilizers exhibit a tendency to be excluded from a protein’s hydration layer, the shell of bound water molecules around the surface. This was termed “preferential exclusion” (Timasheff, 1992), which creates an entropically unfavorable order of high and low solute concentrations and more- and less-ordered water. Proteins reduce this order by minimizing their exposed surface areas by folding more compactly (Fig. 4.10A, middle), especially α -helical secondary structure (Fig. 4.10B). Binding of ligands to active sites will also be favored if this involves loss of ordered bound water (Fig. 4.10A, middle).

Several hypotheses have been and continue to be proposed to explain the forces causing preferential exclusion. Hypotheses relevant to TMAO involve enhanced water structure (Fig. 4.10C), via binding to water molecules better than water does to itself. Evidence for water-structuring effects are numerous. Early examples include osmometry showing that TMAO is a “super-osmolyte” with an osmotic coefficient well above 1.0 (Fig. 4.2C), and a reduction in translational self-diffusion of water in TMAO solutions (Clark, 1985). More recently, atomistic simulations indicate that not only does the oxide end hydrogen-bond more strongly to water than water-to-

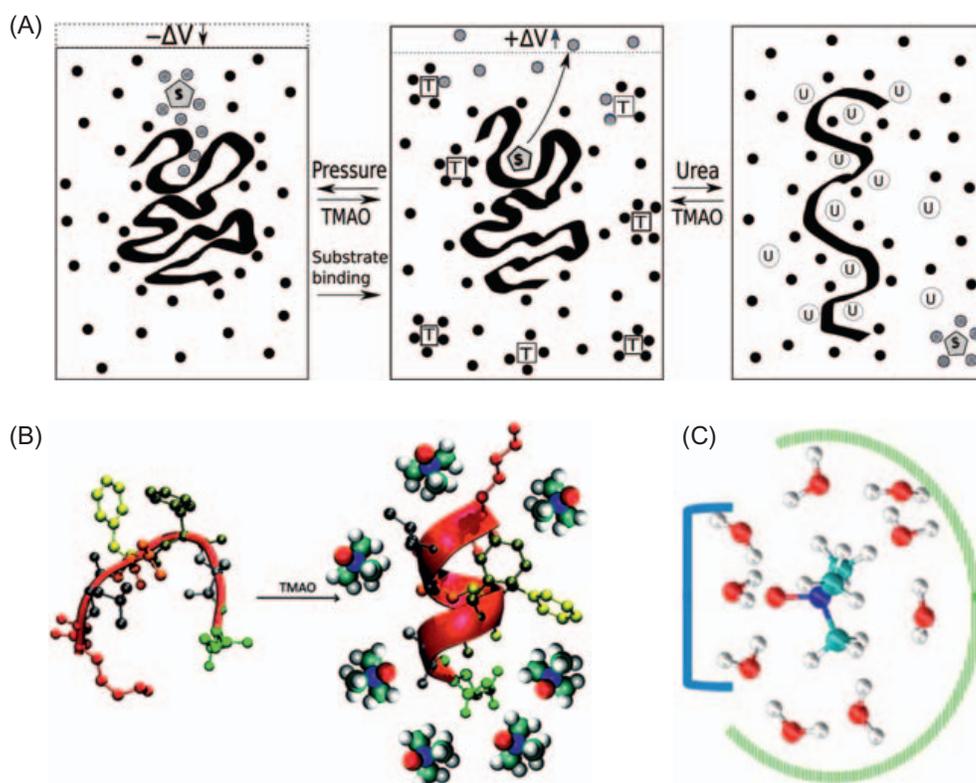


Figure 4.10. Models of TMAO, urea and pressure effects on water and protein folding. Small spheres represent water molecules. (A) Middle panel shows a properly folded protein binding a substrate (S). Bulk water molecules are shown as black spheres. In the right panel, binding of urea (U) to the peptide backbone enhances unfolding since that maximizes favorable binding sites. In the left panel, the substrate S and the protein's active site bind water molecules densely, shown as gray spheres; as those waters are removed during binding, they expand into the bulk phase, causing a positive volume change or $+\Delta V$ in the middle panel. In the left panel, pressure inhibits substrate binding since unbinding involves a volume decrease or $-\Delta V$. Again in the middle panel, addition of TMAO (T), excluded from the protein hydration layer because of its own structured water, favors folding (by preferential exclusion; see main text) and substrate (S) binding (perhaps by aiding removal of dense water from the substrate), counteracting both urea and pressure effects. (B) Model of unfolded protein driven to form an α -helix in TMAO solution. Reprinted with permission from [Cho et al. \(2011\)](#). Copyright 2011 American Chemical Society. (C) Model of water molecules around TMAO molecule (central molecule). Water molecules on the left, indicated by bracket, are held by hydrogen bonds with TMAO oxygen; those on the right, delineated by the $\frac{3}{4}$ circle, are organized in a clathrate structure from TMAO's methyl groups. Reprinted with permission from [Larini and Shea \(2013\)](#). Copyright 2013 American Chemical Society.

water ([Rösgen and Jackson-Atogi, 2012](#); [Doi et al., 2014](#)), but also TMAO's methyl groups induce a restricted water clathrate network as well ([Fig. 4.10C](#)). This enhances local water-water hydrogen bonding but in turn, weakens water's hydrogen bonding to proteins ([Ma et al., 2014](#)).

How do TMAO effects on water structure specifically affect protein folding? Bolen and colleagues have done extensive studies revealing that unfavorable, universal interactions between TMAO-water complexes and peptide backbones (but not amino acid side groups) explain the strong exclusion of TMAO-water, and, therefore, enhancement of protein folding. In essence, the peptide backbone does not dissolve well in TMAO-water solutions compared to pure water, a phenomenon termed “osmophobicity” (Bolen and Bashkakov, 2001; Street et al., 2006). Many osmolytes exhibit osmophobicity, but TMAO is the strongest stabilizer among common osmolytes, as shown in Fig. 4.11.

Recall that urea, in contrast, binds to the peptide backbone (Fig. 4.10A, right). Importantly, for thermodynamics of “dissolving” the peptide backbone in osmolyte solutions, TMAO has a positive G (free energy) – meaning unfavorable – that is about double the negative G for urea – favorable for dissolving (Fig. 4.11). In other words, TMAO’s folding and urea’s unfolding effects with respect to the peptide backbone will cancel thermodynamically about 2:1. This readily explains their universal (de)

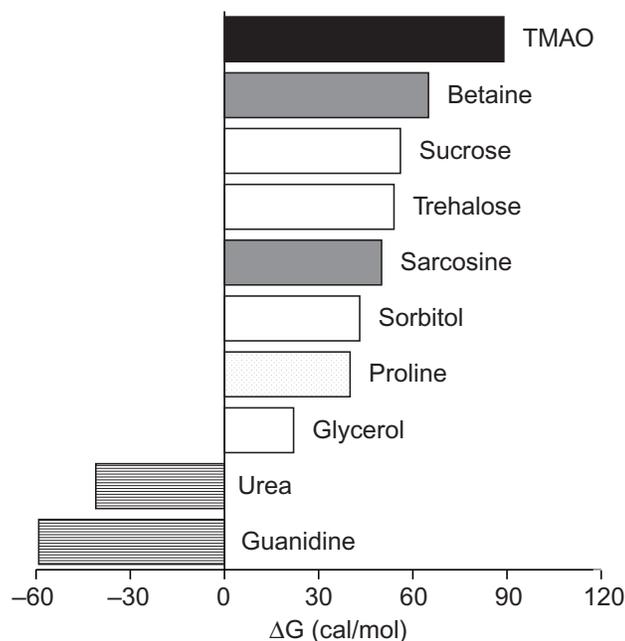
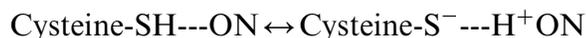


Figure 4.11. Thermodynamic ΔG (cal/mol) of transfer of peptide backbone to 1 M of indicated osmolyte. Black and gray bars on right are methylamines; clear bars are carbohydrates. Denaturants (urea, guanidine; left-hand bars) have negative values, favorable for “dissolving” of peptide backbone which enhances unfolding. Note that TMAO’s positive (unfavorable) value is +89, about double the favorable negative value for urea -41 ΔG ; that is, TMAO’s folding effects are twice as effective as urea’s unfolding effects, thermodynamically canceling about 2:1. Values plotted from Street et al. (2006).

stabilizing roles, with deviations from 2:1 likely due to different side-group interactions with different proteins (Auton et al., 2011).

TMAO's O-N group can apparently induce the formation of protein S-S bonds via hydrogen bonding (—) in the reaction:



This creates a reactive S that bonds with another S, another mechanism of protein stabilization (Brzezinski and Zundel, 1993).

What about other stabilizing osmolytes? Though less well studied at this level, taurine and inositols may have different effects on water structure than do methylamines, and may thus enhance protein stability in different ways (Jackson-Atogi et al., 2013). Perhaps this explains why mixtures of osmolytes differ among tissues (e.g., Fig. 4.4), but this is not understood.

3.5.3. PIEZOLYTE MECHANISM

Water-structuring/osmophobic effects could also explain TMAO's counteraction of hydrostatic pressure (Yancey and Siebenaller, 1999). Hydrostatic pressure inhibits release of hydration water from substrates and the folding and assembly of proteins in cases where volume expansion occurs. TMAO favors the opposite effect; that is, its strong interactions with water may help remove hydration water (Fig. 4.10A, left vs. middle).

Recent biophysical studies by Sarma and Paul (2012, 2013) used model compounds with TMAO to explore pressure-counteraction mechanisms. Under high hydrostatic pressure, the model compound N-methylacetamide becomes increasingly hydrated and water around its nonpolar methyl group becomes compressed. In a protein, pressure could force water molecules into the hydrophobic interior, fostering unfolding. TMAO prevents these hydration changes to the model compound and may therefore protect proteins by stopping pressure forcing of water molecules into protein interiors.

3.5.4. SYNERGISM?

Although most studies have shown that methylamine and urea effects on proteins are largely independent (e.g., Marcelo et al., 2007; Holthauzen and Bolen, 2007), others studies suggest some synergy, as hinted in the 1980 study with skate osmolytes (Yancey and Somero, 1980; Fig. 4.6C). Whether synergistic interactions occur in elasmobranchs has received little additional attention. A model peptide revealed that methylamines and urea might interact synergistically through hydrogen bonding and van der Waals interactions. The result is that methylamines are stronger stabilizers and urea a weaker destabilizer in combination (Meersman et al., 2009; Kumar

and Kishore, 2013). Other studies showed synergistic interactions between stabilizers in pressure inactivation of proteins (Kidman and Northrop, 2005).

3.6. Urea and Methylamines: Buoyancy

Another important osmolyte hypothesis arose from calculations that many organic osmolyte solutions are less dense than seawater (Fig. 4.12; data from Withers et al., 1994b). Density is a function of partial molal volume (PMV) and molecular weight; TMAO, for example, has a PMV that more than offsets the weight it adds to solution, while K^+ has the opposite effect. Withers et al. (1994b) proposed that urea and TMAO were selected as osmolytes in part because these density properties provide buoyancy for chondrichthyans, which lack swim bladders. Overall, TMAO and urea provide significant buoyancy for marine chondrichthyans (an estimated 6 g/L of lift in one shark; Withers et al., 1994a).

However, the buoyancy hypothesis does not explain the 2:1 ratio because urea is invariably more concentrated than TMAO in shallow-living

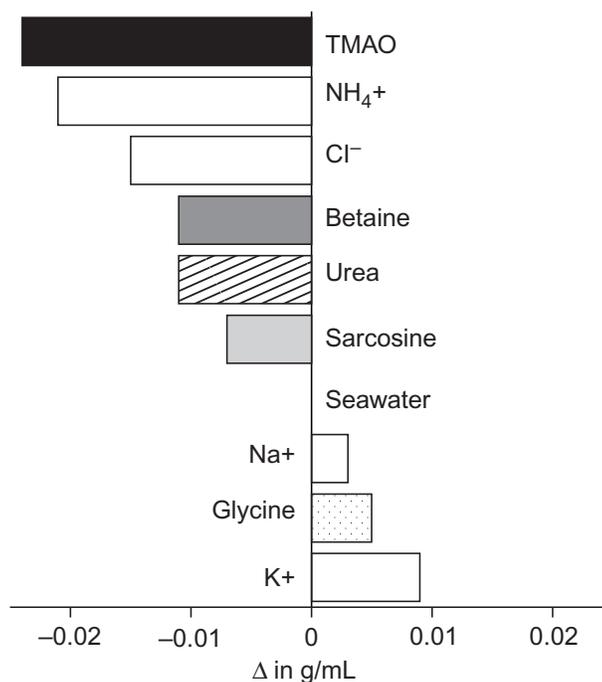


Figure 4.12. Densities of 1 M solutions of the indicated solutes as change (Δ) from average seawater at 1.024 g/mL (set at 0). Negative bars indicate lighter than seawater, positive bars indicate heavier. Solid black and gray negative bars are methylamines; clear bars are inorganic ions. Data plotted from Withers et al. (1994a).

elasmobranchs (Table 4.2), yet urea is less buoyant (denser) than either TMAO (or chloride) (Fig. 4.12). It does not explain osmolyte reduction in euryhaline species that enter waters where buoyancy is more difficult, nor the coelacanth, which has a buoyant swim bladder plus high urea and TMAO at 2:1 (Fig. 4.3). And it does not explain why benthic and pelagic species have almost identical urea and TMAO concentrations (Table 4.2). Withers et al. (1994b) speculate that TMAO is not dominant, despite its buoyancy advantage over urea, because of its high metabolic energy cost for synthesis and/or low availability in the diet. However, TMAO concentrations are much higher in deep-sea skates (Fig. 4.8B) in a food-poor habitat. Similarly, many epipelagic invertebrates (which should benefit from buoyancy aids) can make or accumulate TMAO, but only species in the deep sea have high concentrations (Kelly and Yancey, 1999). It seems likely that the 2:1 ratio is selected for counteraction against each other and with pressure, buoyancy being a secondary factor.

3.7. Other Cytoprotective/Compensatory Properties

Many organic osmolytes also have other roles not related to broad protein (de)stabilization aspects, most of which are beyond the scope of this review (see Yancey, 2005). These properties tend to be unique to each osmolyte type, which may partly explain the complex and different mixtures of osmolytes found among species and organs. The complexity of potential roles led Gilles (1997) to propose the term “compensatory solutes.”

Taurine is perhaps the most complex in terms of possible functions. It is thought to have many (poorly understood) functions: “osmotic pressure, cation homeostasis, enzyme activity, receptor regulation, cell development and cell signaling” and “indirect regulator of oxidative stress” (Schaffer et al., 2010). Some of these roles may explain why taurine concentration is often highest in mammalian and fish hearts and brains (Fig. 4.4C); for instance, 140 mM in skate heart (King and Goldstein, 1983). A few other examples relevant to elasmobranchs are considered next.

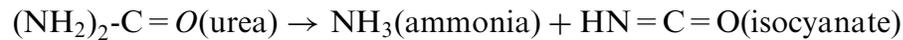
Many osmolytes are thought to have antioxidant properties. This property is commonly attributed to taurine, although its effects are indirect; for instance, it may enhance the electron-transport chain during oxidative stress (Jong et al., 2012). Urea has been found to protect shark and rat heart from oxidative stress, via an ability to scavenge reactive oxygen species (Wang et al., 1999). Betaine and TMAO have antioxidant activity with respect to lipid auto-oxidation (Ishikawa et al., 1978).

As noted earlier, most organic osmolytes can stabilize proteins against thermal denaturation, although again the degree varies by type. Termed by

some as “thermolytes,” such solutes (e.g., di-myo-inositol phosphate) are best known in hydrothermal-vent and hot-spring microbes (reviewed by [Yancey and Siebenaller, 2015](#)). Among eukarya, there is much less evidence for thermolytes. For example, the counteraction between TMAO and hsp70 for shark tissue transport ([Fig. 4.7A](#)) noted earlier, is a good model system for testing TMAO properties, but it is not known if such a function is important in a living elasmobranch. Conversely, TMAO may serve as a colligative antifreeze/cryoprotectant in some Antarctic teleosts (up to 150 mM; [Raymond and DeVries, 1998](#)).

3.8. Implications for Food Industry

Urea is of concern to the fisheries because it breaks down into harmful ammonia, and also isocyanate, which carbamylates proteins, changing the taste and texture of muscle ([Stark, 1972](#)):



However, the relatively high membrane permeability of urea makes it much easier to remove than TMAO during elasmobranch-meat preparation. TMAO is of great concern because it too can break down into harmful products, especially TMA, a volatile base (see [Section 4](#)). This is due to endogenous enzymes or bacteria ([Sotelo et al., 1995](#)). TMA not only has a strong odor of rotten fish, making food unpalatable to most people, but it is also highly toxic; for instance, there is evidence that TMA in Greenland shark meat has poisoned both humans and sled dogs, causing convulsions, vomiting, diarrhea, and even death ([Anthoni et al., 1991](#)). On the other hand, ammonia- and TMA-laden fermented Greenland-shark meat is a delicacy in Iceland ([Herz, 2012](#)). The author can attest first-hand that this must be an acquired taste!

An unexpected implication for food processing has come from the piezolyte discovery. High pressure is increasingly being used to sterilize foods as a less destructive treatment than heating or irradiating. However, some food-poisoning microbes can survive pressure sterilization if they take up methylamine (but not other) osmolyte-type solutes ([Smiddy et al., 2004](#)).

4. METABOLISM AND REGULATION

Clearly, large amounts of organic osmolytes are required for elasmobranch osmotic balance. Metabolism and regulation of elasmobranch osmolytes have been thoroughly reviewed recently ([Hammerschlag, 2006](#);

Trischitta et al., 2012; Gelsleichter and Evans, 2012) including in this book series (Ballantyne and Fraser, 2013), so only a brief version will be given here. (See also Chapters 5 and 7 for more information).

4.1. Osmolyte Synthesis Versus Dietary Intake

Urea, synthesized in part to detoxify ammonia from protein catabolism, is produced primarily in vertebrate livers via the ornithine-urea cycle (OUC). However, unlike most other vertebrates, it starts in elasmobranchs with the amine group of glutamine via carbamoyl phosphate synthase III (Fig. 4.13A, left) rather than free ammonia (Fig. 4.13A, right). Skeletal muscles in several species have OUC enzymes; muscle may contribute about 5% of the urea in the skate (*Leuco-)*Raja erinacea (Steele et al., 2005), and even more from the liver in dogfish shark *Squalus acanthias* (Kajimura et al., 2005). The stomach may also contribute in some species (Tam et al., 2003). The OUC rate is regulated with salinity changes; for example, it increases almost 3-fold in livers of the euryhaline bull shark when transferred from freshwater to seawater (Anderson et al., 2005).

Urea synthesis may also be linked to an unusual feature of elasmobranch energy metabolism. Unlike other vertebrates, elasmobranch skeletal and cardiac muscles (and some other nonhepatic tissues) cannot use fatty acids for fuel. Instead they rely on ketone bodies and amino acids. The latter yield ammonia that can be used by the liver for urea synthesis, as first suggested by Ballantyne (1997). Recent analysis by Speers-Roesch and Treberg (2010) supports the idea that this metabolism evolved for that role (Fig. 4.14).

Synthesis of methylamines is primarily in the liver also (Fig. 4.13B). Betaine is a product of lipid/choline metabolism via betaine aldehyde dehydrogenase (BADH). TMAO synthesis serves in part to detoxify TMA in the diet (e.g., it is high in many squid as a buoyancy aid) or from betaine breakdown (Fig. 4.13B). TMA is converted into TMAO by microsomal TMAO oxidase (TMAOX), an NADPH-dependent enzyme in smooth endoplasmic reticulum (Schlenk, 1998). Mechanisms of methylamine regulation in elasmobranchs are not well known, although synthesis sometimes (but not always) increases in animals transferred from low to high salinity.

TMAO can also be obtained directly from a carnivorous diet, as many prey fish and crustaceans have TMAO. Choline may also be obtained primarily from diet (King et al., 1980) for the betaine pathway (Fig. 4.13B). Recently, Dove et al. (2012) performed metabolomic analysis of healthy and unhealthy whale sharks and found that the former had higher serum levels of the methylamine homarine (N-methylpicolinic acid), and they propose it

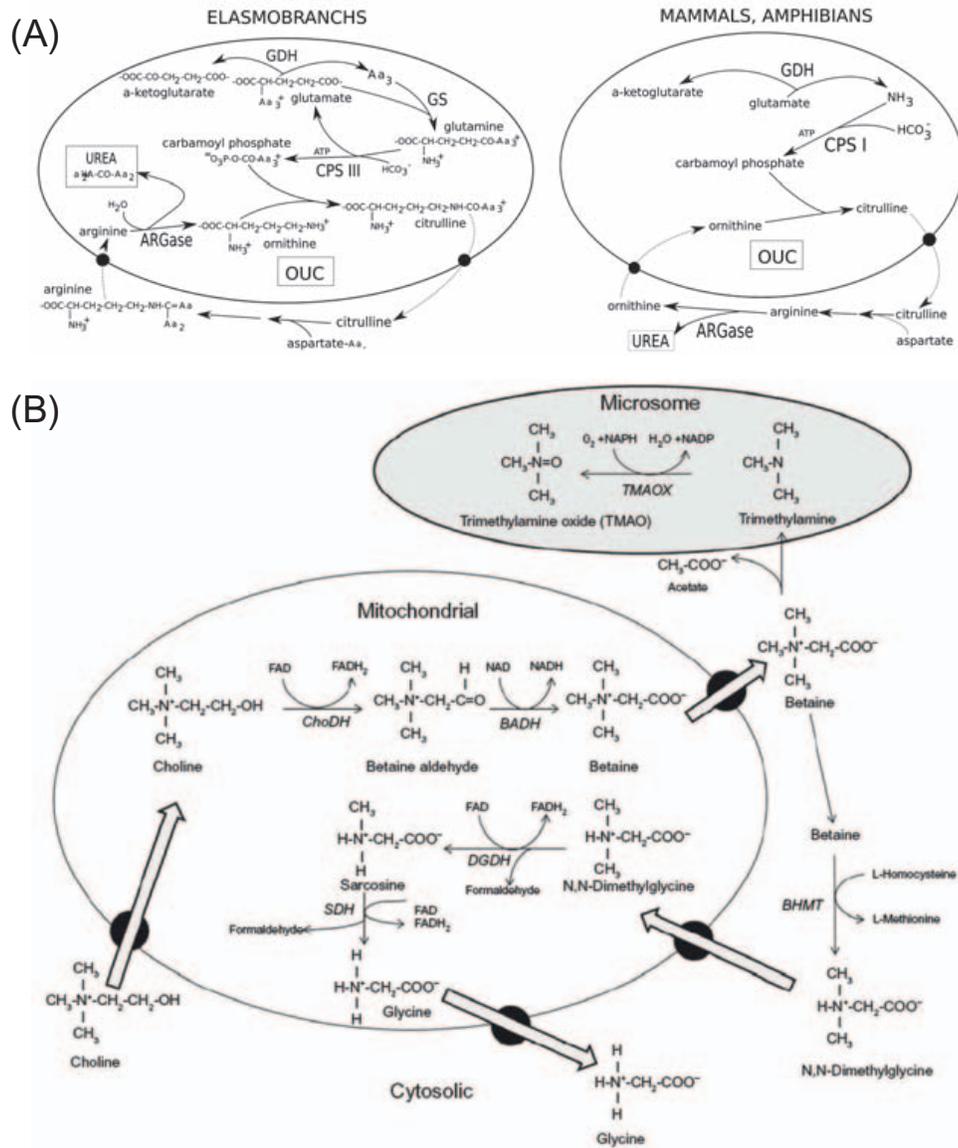


Figure 4.13. Pathways of osmolyte synthesis in elasmobranchs. (A) Urea synthesis via the OUC (ornithine urea cycle), the loop with arginine, ornithine, citrulline and aspartate. Key enzymes are glutamate dehydrogenase (GDH), glutamine synthetase (GS) carbamoyl phosphate synthetase (CPS) and arginase (ARGase). Large ovals represent mitochondria, with small black spheres representing transmembrane transporters. Left side is for elasmobranchs, in which the carbamoyl phosphate that donates an amine group (shown as NH) to ornithine originates from glutamine via CPS III. An additional amine (shown as NH) originates from aspartate. Right side is for mammals and amphibians, simplified without molecular structures, in which the OUC is similar, but carbamoyl phosphate originates from ammonia via CPS I. Adapted from Anderson (1991). (B) Possible pathways for methylamine syntheses. Betaine is produced from choline in mitochondria via the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase, while TMAO is thought to be produced in microsomes from trimethylamine (cleaved from betaine) via TMAO oxidase. Figure courtesy of J.S. Ballantyne.

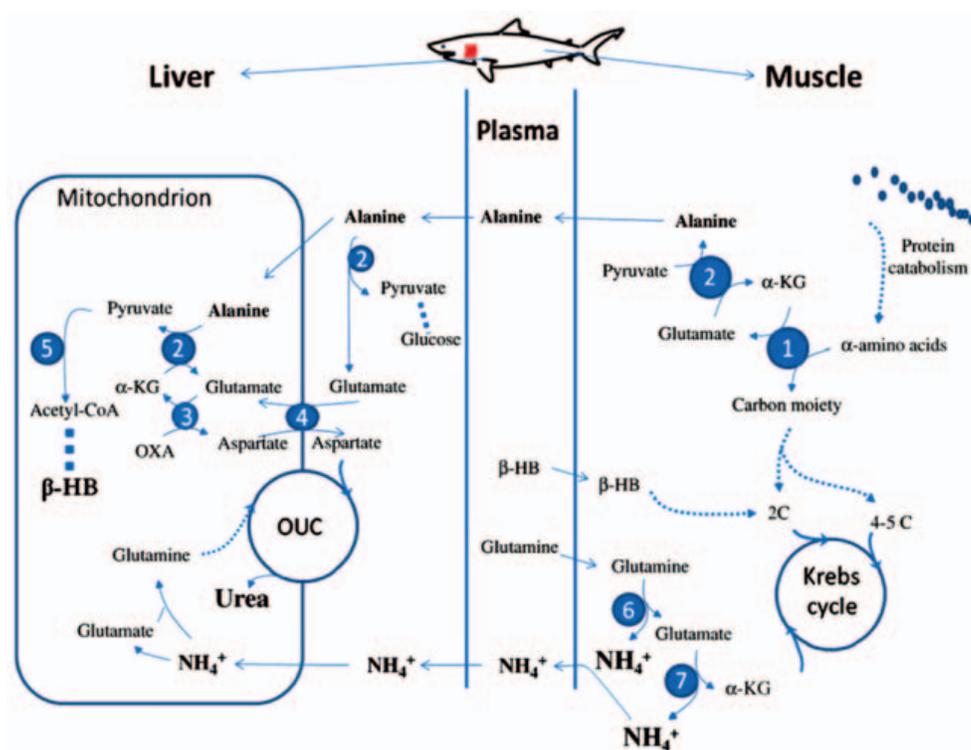


Figure 4.14. Proposed energy metabolism in elasmobranchs. Note the reliance of muscle on protein catabolism (steps 1–2) and glutamine (steps 6–7) for energy and to provide alanine and ammonium for urea synthesis in the liver (steps 2–4 and OUC). The liver also makes ketones (β -HB, β -hydroxybutyrate; step 5) that muscles also use for energy. Enzymes: 1, aminotransferases (general); 2, alanine aminotransferase; 3, aspartate aminotransferase; 4, glutamate aspartate transporter; 5, pyruvate dehydrogenase; 6, phosphate-dependent glutaminase; 7, glutamate dehydrogenase. From Speers-Roesch and Treberg (2010), Copyright 2010 Elsevier.

as biomarker of health. Since homarine has been primarily reported as an osmolyte in crustaceans, the health differences may reflect different dietary intakes.

Yet the sources of TMAO – endogenous synthesis and/or exogenous diet – are not well known for most Chondrichthyes. Some elasmobranchs have TMAOX (Goldstein and Funkhouser, 1972) but others may not and thus require dietary intake (Forster and Goldstein, 1969). However, some species originally reported to lack TMAOX have since been found (with improved methods) to contain it (Schlenk, 1998). Even so, in one modern study (Treberg et al., 2006), a variety of rays had no detectable TMAOX, but rather had high levels of BADH, which is consistent with those species' using betaine more than TMAO (though TMAO was still present). In contrast, while a deep-sea shark (*Centroscyllium punctatum*) had high levels of TMAOX, it had low

levels of BADH, a key part of the pathway to TMAO. One possible reason for the lack of consistency in finding TMAOX is that it could be synthesized in nonhepatic tissues (Treberg et al., 2006), but this has not been studied.

4.2. Retention of Osmolytes

The organs that are most important to urea and TMAO retention – the gills, kidneys rectal gland and gut, which are the potential sites of loss – have known for some time, but details on some functions are still being learned.

4.2.1. GILLS

Most lipid bilayers are impermeable to TMAO since it is charged. Gills are no exception (Shuttleworth, 1988). However, urea permeability, though low for basic membranes, is high enough that unacceptable urea losses could occur. Instead the gills have an unusual lipid composition including extremely high cholesterol content reducing urea permeability, though not completely (at least in dogfish; Fines et al., 2001). The gill apical and/or basolateral membrane may be the limiting site with urea “back-transporters,” possibly sodium-coupled antiporters, that recover some urea as it leaks out (Part et al., 1998; Fines et al., 2001; Evans et al., 2005; Wood et al., 2013). Even so, some urea leaks out for excretion purposes (see Chapter 5).

4.2.2. KIDNEYS

The kidneys reabsorb most of urea and TMAO from the nephron filtrate (Smith, 1936). The renal nephrons are among the most complex known in vertebrates, with 4 loops and countercurrent flows hypothesized to be essential for this reabsorption (Lacy and Reale, 1995). Much more is known about urea than about TMAO recovery mechanisms. The loops express different urea transporters – UTs – that work by facilitated diffusion, probably in conjunction with a countercurrent system and by co-transport with Na^+ (#4 and 5, Fig. 4.15), as in mammalian kidneys (reviewed by McDonald et al., 2006).

In low salinities, urea reduction occurs as the result of either reduced synthesis (Forster and Goldstein, 1976; Tam et al., 2003; Anderson et al., 2005) or a higher renal clearance rate (Goldstein and Forster, 1971; Janech et al., 2006). Filtration rates can increase but there can also be molecular changes: nephron UT expression decreases in skate (*Leuco-*)*Raja erinacea* (Morgan et al., 2003) and shark *Triakus scylium* (Yamaguchi et al., 2009) in dilute salinities, resulting in less urea retention. In the shark UT changes occur in the collecting duct (IV, Fig. 4.15), especially in apical membranes. In concert with the hypothesis of loop function, the nephrons in freshwater

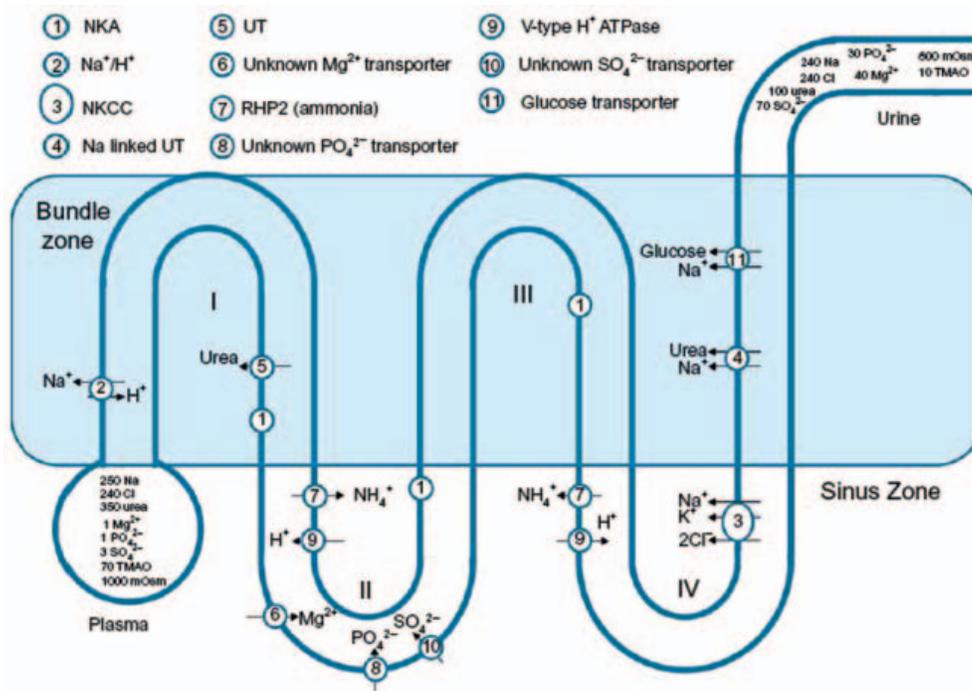


Figure 4.15. Proposed transport functions of nephron loop regions in an elasmobranch kidney. Loops I, II correspond to proximal tubule segment Ia and IIa, respectively, III to early distal tubule and IV to late distal tubule. NKA, Na/K ATPase; NKCC, Na/K/2Cl cotransporter; UT, urea transporter; RHP2, rhesus protein 2. From [Ballantyne and Fraser \(2013\)](#), with permission.

Potamotrygon rays are shorter, with only 2 (less developed) loops, missing the ones linked to urea reabsorption ([Lacy and Reale, 1995](#)).

4.2.3. RECTAL GLAND AND INTESTINES

The rectal gland helps maintain osmotic balance by producing secretions isosmotic with plasma, but with high NaCl and very low urea ([Burger and Hess, 1960](#)) and TMAO. In dogfish the gland as a whole has as a urea content as high as other organs, but may also have a UT to return urea to the blood and keep it out of the filtrate ([Silva et al., 2013](#)). The intestine may also play a role in reabsorbing urea via a transporter ([Anderson et al., 2012](#); [Liew et al., 2013](#)).

4.3. Development

Regulation in marine elasmobranch embryos varies by development type. Ontogeny of osmolyte synthesis is delayed in viviparous species, presumably because maternal intrauterine fluids maintain osmotic balance

(Kormanik, 1993). In the few oviparous species studied, urea and TMAO synthesis arise at the earliest stages (Read, 1968). (*Leuco-*)*Raja erinacea* embryos have OUC enzymes and osmolyte contents that are regulated between 100% and 75% seawater, starting at embryonic age of 4 months when the egg case opens to the environment. The ratio of urea to TMAO and other methylamines was 2.3 to 2.7:1 (Steele et al., 2004), which, as discussed earlier, would be close to 2:1 inside cells.

4.4. Cell Volume Regulation

As noted earlier, osmoconforming cells can volume-regulate on their own. Skate (*Leuco-*) *Raja erinacea* erythrocytes maintain amino acid osmolytes and TMAO at high levels, at least some via Na⁺-dependent transporters (King and Goldstein, 1983; Wilson et al., 1999). These erythrocytes reduce organic osmolytes in hypotonic stress via membrane channel proteins (McConnell and Goldstein, 1988; Kooma et al., 2001) similar to mammalian multifunctional volume-regulated anion channels recently identified (Voss et al., 2014) that release organic osmolytes (Fig. 4.16). Proteomic analysis of leopard-shark rectal-gland and gill cells exposed to low salinities revealed (among other things) changes in osmolyte metabolism, including enhanced myo-inositol (Fig. 4.4C) degradation (Dowd et al., 2010). However, regulatory mechanisms are mostly unknown. See Ballantyne and Fraser (2013) for a recent review.

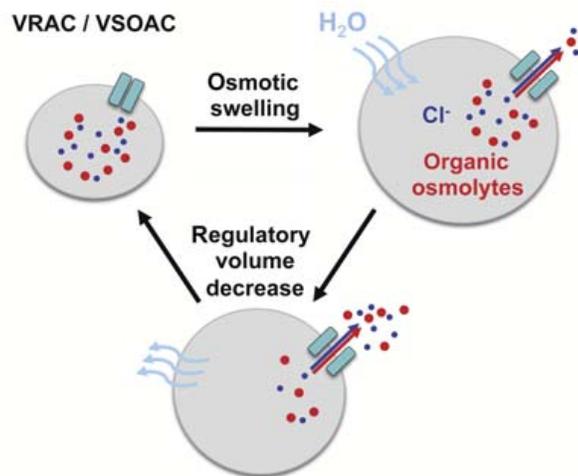


Figure 4.16. Volume-regulated anion channel (VRAC) recently identified in mammals by Voss et al. (2014). Similar channels have been found in elasmobranch cells (see text). Figure used with permission courtesy of Felizia Voss and Thomas Jents.

4.5. Hormonal Regulation

Elasmobranchs have a variety of hormones involved in osmotic balance through four major systems (similar to those of other vertebrates), reviewed recently by [Gelschlechter and Evans \(2012\)](#): (i) the *hypothalamus-pituitary-interrenal* axis; (ii) the *renal renin-angiotensin* system; (iii) the *rectal gland* and its vasoactive intestinal peptide (VIP); and (iv) the *heart* and its C-type natriuretic peptide (CNP). However, while these have been found to regulate water and inorganic osmolyte balance, little is known about regulation of organic osmolytes. See Chapter 8 for more on elasmobranch hormones.

5. EVOLUTIONARY CONSIDERATIONS

Possible scenarios for the evolution of fish osmoregulation/osmoconformation have been presented many times (reviewed by [Schultz and McCormick, 2013](#)). Primitive kidney features suggest that protovertebrates evolved in low salinities and/or as euryhaline ([Griffith, 1987](#); [Ditrich, 2007](#)). For teleosts, strong physiological, fossil, and (most recently) phylogenetic/molecular evidence indicates that ancestors evolved in freshwater or estuaries ([Vega and Wiens, 2012](#)). For stability in varying hypo-osmotic environments, these fish may have evolved osmoregulatory homeostasis, especially useful for later invading seawater. Teleost osmoregulation is thought to be costly, using 15% or more of their aerobic “energy budget” ([Griffith and Pang, 1979](#); reviewed by [Febry and Lutz, 1987](#)), and so it has been speculated such regulation could not have arisen before atmospheric oxygen reached a high enough level, perhaps 300 million years ago ([Ballantyne et al., 1987](#)). The benefit lies in an internal environment largely independent of the external, much like the costly thermoregulation of birds, mammals, tunas, etc., “pays off” in protection of internal systems from thermal disturbances. Like mammals spreading to most thermal habitats, teleosts have readily invaded a greater range of salinities than elasmobranchs.

What about elasmobranchs? The hypoionic state of marine elasmobranchs suggests that their ancestors also lived in low salinities. Evidence is unclear: fossil proto-sharks from almost 400 million years ago are mostly marine, but one prominent one, *Xenacanthus*, was freshwater ([Klimley, 2013](#)). Urea may have been “selected” first for ammonia detoxification and excretion, perhaps for estivation in ephemeral inland waters ([Griffith, 1991](#)). Urea’s suppressive effects are useful in estivation ([Yancey, 2005](#)), but perhaps at some point, invasion of seawater began and urea became retained as an osmolyte, which necessitated methylamine retention at the same time. However it originated, the elasmobranch-type OUC seems to have arisen

before the elasmobranchs (Mommensen and Walsh, 1989). Alternatively, it is possible that the urea/TMAO system first evolved in seawater, perhaps needed within an egg capsule with a long development (Griffith, 1991), or as buoyant replacements for heavy plasma ions in the absence of swim bladders (McNab, 2002). TMAO may have arisen as a byproduct of high lipid metabolism involved in other functions such as buoyancy (Seibel and Walsh, 2002), but it is not clear why so many elasmobranchs appear to be unable to make TMAO. Regardless of origin and unlike teleosts, the urea/TMAO system has made it difficult for elasmobranchs to enter (or return to) low salinities. Using compatible or counteracting osmolytes that are regulated to conform with a changing environment should, in theory, allow proteins to maintain proper function over a wide range, but this cannot work at low salinities.

Osmoconforming's one advantage has long assumed to be less cost, in part because water balance itself costs no energy (Griffith and Pang, 1979; Ballantyne et al., 1987), but this is by no means certain. Urea synthesis costs 5 ATPs per molecule, and TMAO synthesis uses energy of NADPH; Kirschner (1993) calculated that osmoconforming with urea could cost up to 15% of the aerobic energy budget, possibly just as costly as teleost osmoregulation.

6. KNOWLEDGE GAPS AND FUTURE DIRECTIONS

Many questions remain unanswered regarding elasmobranch organic osmolytes, including the following:

- The evolutionary origins of elasmobranch osmotic physiology
- Genes, RNAs (including microRNAs), and proteins (membrane channels, synthetases, etc.) for regulation of cellular organic osmolytes: mechanisms and regulation
- Hormonal and intracellular regulation of plasma organic osmolytes and synthesis
- Detailed mechanisms of osmolyte properties including co-evolution of protein intrinsic structures with osmolytes, proteins under pressure, possible synergies, unique properties of each class of osmolyte and why they differ among organs and species. For example, why is rectal gland but not other organs high in scyllo-inositol (Fig. 4.4C), a stabilizer currently in clinical trials to inhibit amyloid- β formations in Alzheimer's Disease (Sun et al., 2008)?

Some answers will require more use of genomics, proteomics, and metabolomics. Elasmobranch osmotic adaptations will continue to be medical

models, especially since the discovery of elasmobranch urea-TMAO counteraction led to the finding of similar counteraction in mammalian kidneys. As a different example, [Feige et al. \(2014\)](#) used the urea-resistance features of shark Ig to engineer a human Ig that was also more stable, possibly improving shelf life and efficacy in patients. Protective properties of osmolytes will continue to receive attention. Since such properties may be universal in their effects, they can be applied to medical, agricultural, and biotechnological needs. For example, several groups (e.g., [Welch and Brown, 1996](#); [Zhao et al., 2007](#)) have suggested that osmolytes as chemical chaperones might rescue misfolded proteins in human diseases. Stabilizing biochemical preparations is another example. Examples are shown in [Table 4.5](#).

In conclusion, basic research on the osmotic strategies of elasmobranchs has not only yielded important insights regarding biochemical evolution of cellular milieus, but has also contributed significantly to biomedical and other applied biosciences. We can expect more such discoveries through continuing work on this fascinating group of fishes.

Table 4.5
Examples of potential or actual applications of TMAO

| Biochemical procedure tested and effect of TMAO | Reference |
|---|---|
| Prion diseases: damaging scrapie and bovine spongiform encephalopathy formations inhibited by TMAO <i>in vitro</i> | Tatzelt et al. (1996) and Bennion et al. (2004) |
| Alzheimer's Disease: amyloid- β inhibited by TMAO <i>in vitro</i> | Yang et al. (1999) |
| Maple syrup urine disease: function of mutated protein causing disease is restored by TMAO <i>in vitro</i> | Song and Chuang (2001) |
| Cystic fibrosis: folding and function of the mutated channel in cultured cells promoted by TMAO and other osmolytes | Howard et al. (2003) |
| Lysosomal storage disorder: oxidative stress responses in cultured cells alleviated by TMAO | Wei et al. (2008) |
| Glaucoma: cured in human patients given TMAO injections | Jia et al. (2009) |
| Cataracts: prevented in lens cell culture by TMAO | Gong et al. (2009) |
| Type-II diabetes: formation of amyloidogenic islet amyloid polypeptide inhibited by TMAO <i>in vitro</i> | Seeliger et al. (2013) |
| Asthma: unfolded proteins in mice prevented by TMAO | Makhija et al. (2014) |
| <hr/> | |
| Biochemical procedure tested and effect of TMAO | |
| Protein encapsulation for biochemical studies: enhanced by TMAO | Glasgow et al. (2012) |
| Cartilage tissue engineering: improved mechanical and biochemical properties with TMAO up to 100 mM | O'Connell et al. (2012) |
| X-Ray crystallography: crystal formation of proteins enhanced by TMAO | Marshall et al. (2012) |

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REGULATION OF IONS, ACID–BASE, AND NITROGENOUS WASTES IN ELASMOBRANCHS

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Elasmobranch fishes osmoconform to the marine environment by synthesizing and retaining substantial levels of urea (~350 mM) and other organic osmolytes. Urea is reabsorbed at the kidney and although renal urea transport proteins have been isolated and characterized there is still a limited understanding of the reabsorptive processes involved. Marine elasmobranchs are nitrogen-limited and attention has now turned to ammonia retention mechanisms. Elasmobranch fishes regulate plasma ions below seawater levels but above what is typical of marine teleosts. The gill plays a primary role in ion regulation, with the kidney and rectal gland contributing as well. The gill also has a phenomenal capacity to eliminate acid or base loads. Recent discoveries of solute transporters in the gill, kidney, and gut of elasmobranchs bring an enhanced understanding of ion and acid–base regulation. There have been a series of exciting papers on the elasmobranch gut that demonstrate a dynamic tissue with multiple roles. This chapter

discusses recent and earlier research on ion, acid–base, and nitrogen regulation in these magnificent fishes inhabiting marine, fresh water, and intermediate salinity environments. There is tremendous scope for novel discoveries in this captivating group of fishes and we have identified compelling research questions for fish biologists.

1. INTRODUCTION

Elasmobranchs balance ions and water in unique and fascinating ways. Although most species are marine there are some interesting exceptions that tolerate or thrive in dilute environments. Marine elasmobranchs are osmoconformers, but ionoregulators. It is an intriguing physiological paradox that ions and organic osmolytes in body fluids are both carefully regulated, yet they conform with respect to osmolyte concentration not composition. Urea is the major organic osmolyte, first discovered in 1858 by [Stäedeler and Frérichs \(1858\)](#) who found “colossal quantities” of urea in muscle tissue of several marine species. Urea is a perturbing solute and years of biochemical work have explored how elasmobranch proteins function in such a harsh environment ([Yancey et al., 1982](#); see also Chapter 4). From an evolutionary perspective it is interesting that marine elasmobranchs are the only vertebrate group with a rectal gland, a small excretory organ that delivers a salt-rich fluid into the lower gut. Some of these unique characteristics of elasmobranchs have been well reviewed by others, but we add new perspectives on the older literature and review more recent findings.

2. IONOREGULATION

Previous reviews on various aspects of ionic and osmotic regulation in elasmobranchs include [Thorson \(1967\)](#), [Forster \(1967\)](#), [Hickman and Trump \(1969\)](#), [Pang et al. \(1977\)](#), [Shuttleworth \(1988\)](#), [Henderson et al. \(1988\)](#), [Hazon et al. \(1997\)](#), [Lacy and Reale \(1995\)](#), [Olson \(1999\)](#), [Hazon et al. \(2003\)](#), [Evans et al. \(2004, 2005\)](#), [Hammerschlag \(2006\)](#), [Anderson et al. \(2007\)](#), [Ballantyne and Robinson \(2010\)](#), and [Ballantyne and Fraser \(2013\)](#). The golden age of studies on ionoregulation in elasmobranchs was from the 1960s to the 1980s by researchers who picked up the mantle from the pioneering studies of Homer Smith 30–50 years earlier ([Smith, 1929, 1931a,b,c, 1936](#)). Unfortunately, much of this brilliant early work has been forgotten or overlooked by modern investigators, whose focus has been

more on the linkages of acid–base regulation to ionoregulation and their molecular mechanisms than on ionoregulation itself. One of our goals is to highlight some of these innovative early studies and, in particular, correct some current misconceptions about the dominance of the rectal gland and lack of importance of the gills and kidney in normal ionoregulation in seawater.

2.1. Overview

Marine elasmobranchs maintain the osmolality of their internal fluids slightly higher than that in the external seawater ($\sim 1000 \text{ mOsmol L}^{-1}$), greatly reducing the need for drinking to obtain free water. This strategy is very different from the drinking-based approach of marine teleosts, which obligates the need for active branchial excretion of the NaCl taken up with water at the gut; overall the two strategies are probably about equal in metabolic cost (Kirschner, 1993). In marine elasmobranchs, most of the extracellular osmolality is contributed by these two electrolytes (Na^+ and Cl^-), which together account for about 50–60%, and two nonelectrolytes, urea and trimethylamine oxide (TMAO), which account for about 30–40% and 5–10% respectively. Plasma Na^+ and Cl^- concentrations are typically around 250–300 mmol L^{-1} , at least 100 mmol L^{-1} higher than in teleosts, but still only about half of those in the external seawater, so there are still gradients for Na^+ and Cl^- entry at the gills. There are even stronger gradients for the entry of K^+ , Ca^{2+} , Mg^{2+} , and SO_4^{2-} because their concentrations are regulated at very low values (a few mmol L^{-1}) in blood plasma relative to the high levels in external seawater. Evans et al. (2004) and Hammerschlag (2006) provide useful summary tables for all these parameters. The extracellular fluid volume (ECFV) comprises only about one fifth of the body water volume (relatively small for vertebrates), and in the much larger intracellular fluid volume (ICFV), the osmotic roles of Na^+ and Cl^- are largely replaced by K^+ and organic anions. Intracellular and extracellular urea concentrations are similar, but intracellular TMAO levels may be up to 5-fold higher than extracellular. In this section we focus on the regulation of ions and water; see Section 4.0 for discussion of urea, TMAO, and other organic anions.

The organs of ionoregulation and water balance are the gills, gut, kidney, and rectal gland, a small digitiform organ found only in cartilaginous fishes and coelocanths. The gland is packed with mitochondria-rich cells (MRCs) and can discharge an almost pure 0.5M NaCl solution into the colon for excretion (reviewed by Olson, 1999). While it is often claimed that this is the major organ of NaCl excretion, we are aware of no definitive evidence that this is true. It has been shown numerous times that when the rectal gland is ligated or surgically removed, elasmobranchs are still able to

maintain normal concentrations of Na^+ and Cl^- in the blood plasma. In fasting animals, the gills probably account for the majority of normal Na^+ and Cl^- regulation, with lesser contributions by the kidney and rectal gland. The latter appears to be called into action especially at times of salt or volume loading, such as feeding and/or ingestion of seawater. Additionally, the gills are the major site of water uptake during fasting, and although critically important in urea and TMAO retention, they are the major site of loss of these key osmolytes (see [Section 4.1.1](#)). Drinking is slight but finite, and the gut's major osmoregulatory role is probably in the uptake of water from hypotonic food (e.g., teleost prey), the recycling of urea, and in the exclusion of toxic divalent ions (Ca^{2+} , Mg^{2+} , and SO_4^{2-}). The kidney probably plays a major role in the excretion of these toxic divalents as well as inorganic phosphate, and in the retention of urea and TMAO, but only a modest role in NaCl excretion as it appears to be unable to secrete a fluid hypertonic to the blood plasma.

Only 10% of all elasmobranchs are estuarine, 1% are truly euryhaline, and 2–3% are obligate in freshwater ([Martin, 2005](#)). As elasmobranchs enter more dilute environments, they cannot osmoconform, but nevertheless, they reduce internal osmolality by dumping urea, TMAO, and ions at the kidney, and decreasing the metabolic production of urea. Obligate freshwater elasmobranchs have lost the ability to make urea by the ornithine-urea cycle (OUC) and to retain it at the kidney, and they maintain plasma Na^+ , Cl^- , divalent ions, urea, and TMAO all at levels similar to those of freshwater teleosts. Ostensibly, they osmo- and ionoregulate like freshwater teleosts, with active ion uptake at gills and gut, minimal drinking, water excretion but ion retention at the kidney, and negligible rectal gland function (reviewed by [Ballantyne and Robinson, 2010](#); [Ballantyne and Fraser, 2013](#); see [Sections 2.3 and 4.3](#)).

2.2. Seawater

2.2.1. GILLS

The normal transepithelial potential (TEP) across the gills of intact elasmobranchs in full strength seawater is close to 0 mV ([Bentley et al., 1976](#); [Evans, 1980, 1982](#); [Evans et al., 1982](#); [Evans and More, 1988](#); [Nawata et al., 2015a](#)) so chemical, rather than electrical gradients largely dictate transepithelial fluxes. [Holmes and Donaldson \(1969\)](#) compiled a detailed summary table of plasma electrolyte concentrations, and these have been confirmed by many more recent measurements (e.g., [Cooper and Morris, 1998](#); [Pillans and Franklin, 2004](#); [Wood et al., 2007b](#); [Kajimura et al., 2008](#)) and summaries ([Evans et al., 2005](#); [Hammerschlag, 2006](#)). Note however

that many reported values of plasma K^+ are probably artificially high due to slight hemolysis during sampling. Plasma Na^+ and Cl^- concentrations (240–300 $mmol L^{-1}$) are typically about 45–55% of those in seawater ($Na \sim 480$, $Cl^- = 560 mmol L^{-1}$) so there are only 2-fold chemical gradients driving the passive gain of Na^+ and Cl^- . Relative chemical gradients (3–30-fold) for the entry of other important ions from seawater ($K^+ \sim 10 mmol L^{-1}$, $Ca^{2+} \sim 10 mmol L^{-1}$, $Mg^{2+} \sim 54 mmol L^{-1}$, $SO_4^{2-} \sim 29 mmol L^{-1}$) are somewhat greater. Plasma concentrations of all of these important electrolytes are normally regulated in the range of 1–5 $mmol L^{-1}$. However, the great majority of experimental work has focused on the regulation of Na^+ and Cl^- , and there is a pressing need for investigations on the regulation of these other ions.

A recent, otherwise excellent (and therefore unnamed) paper stated “Unlike the gills of teleost fishes which transport ions for both acid–base regulation and ionoregulation, the gills of marine elasmobranchs transport ions exclusively for acid–base regulation.” This is doubtful, but reflects a current and common misconception in the field. The rectal gland is often thought to be the major organ of $NaCl$ excretion, but it has been shown repeatedly that when this gland is disabled by removal or ligation, the fish is still able to regulate plasma Na^+ and Cl^- concentrations at normal levels (Burger, 1962, 1965; Chan et al., 1967; Haywood, 1975a,b; Evans et al., 1982; Wilson et al., 2002). Indeed this remains true even when urinary excretion is also blocked (Haywood, 1975b)! The gill is therefore fully functional in ionoregulation. Unidirectional flux measurements with radiotracers ($^{22}Na^+$, $^{36}Cl^-$) have shown that there is a dynamic bi-directional exchange of both ions at the gills. While the data are limited (Table 5.1), they demonstrate that under control conditions (i) the unidirectional influx rates of both Na^+ and Cl^- at the gills exceed their unidirectional efflux rates, such that the excess must normally be excreted through the rectal gland and/or kidney; (ii) the branchial efflux rates still account for about two-thirds of the total efflux rates, so the gill is the major Na^+ and Cl^- excretory organ; (iii) the unidirectional flux rates of Cl^- are 3 to 10-fold greater than those of Na^+ though the reason for this difference is unknown; (iv) the magnitudes of these rates are far in excess of those needed for acid–base regulation, and indeed, as explained subsequently, the Na^+ and Cl^- fluxes needed for acid–base regulation are unidirectional influxes, not effluxes; (v) overall unidirectional exchange rates are above the high end of the range ($<200 \mu mol kg^{-1} h^{-1}$) for freshwater teleosts of comparable size, but below the low end of the range ($>3000 \mu mol kg^{-1} h^{-1}$) seen in marine teleosts of comparable size. The fluxes are lower in marine elasmobranchs than in marine teleosts because the seawater-to-plasma gradients are lower, the permeability of the gill is lower (Evans, 1980), and there is no complicating exchange diffusion component (cf. Motais et al., 1966). At least in *Squalus*

Table 5.1

Rates of Na⁺ and Cl⁻ influx (positive values) and outflux (negative values) through the whole body, gills, rectal gland, and urine in elasmobranchs acclimated to full strength seawater. Values are $\mu\text{mol kg}^{-1} \text{h}^{-1}$

| Species | Total Na ⁺ influx | Total Na ⁺ outflux | Gill Na ⁺ outflux | Urinary Na ⁺ outflux | Rectal gland Na ⁺ outflux | Total Cl ⁻ influx | Total Cl ⁻ outflux | Gill Cl ⁻ outflux | Urinary Cl ⁻ outflux | Rectal gland Cl ⁻ outflux | Reference |
|------------------------------------|------------------------------|-------------------------------|------------------------------|---------------------------------|--------------------------------------|------------------------------|-------------------------------|------------------------------|---------------------------------|--------------------------------------|---|
| <i>Squalus acanthias</i> | +945 | -890 | -740 | -150 | | | | | | | Burger and Tosteson (1966) Burger (1965) Horowicz and Burger (1968) Burger and Hess (1960) Evans et al. (1982) Burger (1967) Schmidt-Nielsen et al. (1972) Benyajati and Yokota (1990) |
| | +680 | | -160 | | (-235) | | | -110 | | -235 | |
| | | -455 | | | -240 | | | | | (-240) | |
| | | | | -120 | | | | -2190 | | | |
| | | | | -115 | | | | | -120 | | |
| | | | -101 | | | | | | | | |
| <i>Scyliorhinus canicula</i> | +1330 | | -290 | | | +4230 | | -2320 | | | Bentley et al. (1976) Maetz and Lahlou (1966) Payan and Maetz (1973) Payan and Maetz (1970) Henderson et al. (1988) Wells et al. (2002) |
| | +560 | | -690 | -105 | | | | | | | |
| | +400 | -220 | | -200 | -20 | | | | | | |
| | | | | -45 | | | | | -69 | | |
| | | | | -18 | | | | | -18 | | |
| <i>Hemiscyllium plagiosum</i> | | -750 | -694 | -56 | -46 | | | | | (-46) | Chan et al. (1967) Chan et al. (1967) Wong and Chan (1977b) |
| | | -750 | -694 | -56 | -46 | | | | | (-46) | |
| | | | -90 | | -235 | | | | -81 | -227 | |
| <i>Poroderma africanum</i> | | -185 | | | | | -2080 | | | | Haywood (1974) Haywood (1974a) |
| | | | | -18 | -32 | | | | -24 | -31 | |
| <i>Raja ocellata</i> | | | | | | | | | | | Holt and Idler (1975) Goldstein and Forster (1971a) |
| | | | | | -310 | | | | | -320 | |
| | | | | | | | | | -34 | | |
| <i>Dasyatis sabina</i> | | | | | | | | | | | Beitz (1977) Janech et al. (2006b) |
| | | | | -104 | (-76) | | | | -83 | -76 | |
| | | | | | | | | | | | |
| <i>Dasyatis pastinica</i> | | | | -22 | -26 | | | | | (-26) | Fleishman et al. (1986) |
| <i>Heterodontus portusjacksoni</i> | | -4180 | | | | | | | | | Cooper and Morris (2004) |
| <i>Ginglymostoma cirratum</i> | | -350 | | | | | -1020 | | | | Carrier and Evans (1972) |

Note: Values in parentheses are based on the assumption that Na⁺ and Cl⁻ concentrations in rectal gland secretion are normally equal.

acanthias, less than 10% of the unidirectional Na^+ fluxes occur through the skin (Horowicz and Burger, 1968).

The seminal radiotracer study remains that of Bentley et al. (1976) who concluded that active Cl^- extrusion likely occurs at the elasmobranch gill, as in marine teleosts, albeit at lower rates. This finding, which should be confirmed, suggests that elements of the classic “Silva” model for active excretion of Na^+ and Cl^- at the marine teleost gill (and elasmobranch rectal gland; Silva et al., 1977a,b) may apply at the marine elasmobranch gill. Certainly, the gill contains relatively high levels of Na^+ , K^+ -ATPase (NKA) activity (Jampol and Epstein, 1970; Morgan et al., 1997; Piermarini and Evans, 2000; De Boeck et al., 2001, 2007; Wilson et al., 2002; Pillans et al., 2005). Although the *specific* activity of NKA is much greater in the rectal gland, *total activity* is much greater in the gills because of their larger size. For example, ouabain-sensitive O_2 consumption of the whole gill was 4 times higher than of the whole rectal gland in *Squalus acanthias* (Morgan et al., 1997). Most of this NKA activity appears to be localized in MRCs, of which there are several different morphologies and functional types (Doyle and Gorecki, 1961; Hughes and Wright, 1970; Wright, 1973; Haywood, 1975a; Laurent and Dunel, 1980; Crespo et al., 1981; Crespo, 1982; Piermarini and Evans, 2000, 2001; Piermarini et al., 2002; Wilson et al., 2002; Tresguerres et al., 2005, 2006; Tresguerres et al., 2007; Reilly et al., 2011; Roa et al., 2014). Principal morphological differences from marine teleost MRCs appear to be the absence of accessory cells next to MRCs, and the absence of an extensive intracellular basolateral tubular system. The latter is replaced by a basolateral membrane, which is heavily folded into a basal labyrinth that bears the NKA. Fig. 3.12 of Wegner (2015) provides a representative transmission electron micrograph. There is probably excess capacity because MRC numbers, morphology, and branchial NKA activity did not increase after removal of the rectal gland in *Squalus acanthias* (Wilson et al., 2002).

One of these MRC types has a dominant basolateral NKA activity and appears to be involved in apical acid excretion coupled to Na^+ influx from the water, and another has dominant basolateral H^+ -ATPase activity and appears to be involved in apical base excretion coupled to Cl^- influx from the water. These mechanisms become prominent during acid-base disturbances, which result in increased influxes of Na^+ (during acidosis) and probably of Cl^- (during alkalosis), processes which actually work against the ion-excretory function of the gill. The physiological and molecular evidence supporting these ideas is summarized in Section 3.2.1.

The other key osmoregulatory function of the gill is to ensure sufficient free water uptake to serve the needs of urinary and rectal gland flow, which together may be up to $2 \text{ ml kg}^{-1} \text{ h}^{-1}$, of which very little comes from drinking (Table 5.2). In view of the small osmotic gradient from seawater to

Table 5.2

Rates of diffusive water exchange (% of body water pool per hour) measured with $^3\text{H}_2\text{O}$, and rates of water influx (positive values) by drinking and outflux (negative values) through the rectal gland and urine in elasmobranchs acclimated to full strength seawater. Influx and outflux rates are in $\text{ml kg}^{-1} \text{h}^{-1}$

| Species | Water turnover rate (% h^{-1}) | Drinking rate ($\text{ml kg}^{-1} \text{h}^{-1}$) | Rectal gland flow rate ($\text{ml kg}^{-1} \text{h}^{-1}$) | Urinary flow rate ($\text{ml kg}^{-1} \text{h}^{-1}$) | Reference |
|-------------------------------|--|---|--|---|-------------------------------|
| <i>Scyliorhinus canicula</i> | 157% | + 0.125 | -0.081 | -0.285 | Payan and Maetz (1971) |
| | | | | -0.305 | Payan and Maetz (1971) |
| | | | | -0.170 | Brown and Green (1987) |
| | | | | -0.190 | Henderson et al. (1988) |
| | | | | -0.082 | Wells et al. (2002) |
| | | | | +0.280 | Hazon et al. (1989) |
| | +0.100 | Anderson et al. (2001) | | | |
| | +0.120 | Anderson et al. (2002b) | | | |
| <i>Squalus acanthias</i> | | | -0.470 | -0.814 | Forster and Berglund (1957) |
| | | | | -0.450 | Burger (1962) |
| | | | | -0.508 | Burger (1962) |
| | | | | -0.280 | Schmidt-Nielsen et al. (1972) |
| | | | | -0.965 | Forster et al. (1972) |
| | | | | -0.484 | King and Goldstein (1983) |
| | | | | -0.300 | Benyajati and Yokota (1990) |
| | | | | -0.210 | Wood et al. (1995) |
| | +0.163 | De Boeck et al. (2001) | | | |
| <i>Poroderma africanum</i> | 97% | | -0.029 | -0.096 | Haywood (1975b) |
| | | | | Haywood (1975a) | |
| <i>Mustelis canis</i> | | | | -0.630 | Kempton (1953) |
| <i>Raja erinacea</i> | 64% | | | -0.190 | Goldstein and Forster (1971a) |
| | | | | -0.291 | Payan et al. (1973) |
| <i>Raja ocellata</i> | | | | -0.640 | Holt and Idler (1975) |
| <i>Raja montagu</i> | 167% | | | | Payan and Maetz (1971) |
| <i>Torpedo marmorata</i> | 97% | | | | Payan and Maetz (1971) |
| <i>Hemiscyllium plagiosum</i> | | | | -0.305 | Chan et al. (1967) |
| | | | | -0.510 | Wong and Chan (1977) |
| <i>Dasyatis sabina</i> | | | | -0.130 | Beitz (1977) |
| | | | | -0.900 | Janech et al. (2006b) |
| <i>Triakis scyllia</i> | | +0.520 | | | Anderson et al. (2001) |
| <i>Ginglymostoma cirratum</i> | 81% | | | | Carrier and Evans (1972) |

plasma (10–70 mOsmol L⁻¹), osmotic permeability must be high. We are aware of only two attempts to determine the osmotic permeability coefficient directly; the values obtained in *Scyliorhinus canicula* (Payan and Maetz, 1971) and *Raja erinacea* (Payan et al., 1973) were about 50-fold and 5-fold higher respectively than in marine teleosts. However, there are several reports of diffusive water turnover rates (measured with ³H₂O) in elasmobranchs; these are in the order of 100% per hour or more (Table 5.2), at least 5-fold greater than in seawater teleosts. Only a small fraction (<15%) of this water flux occurs across the skin (Payan and Maetz, 1971). How does the elasmobranch gill achieve such high water permeability yet keep permeability to ions and urea so low? The answer may lie in aquaporins. Aquaporin AQP4 (the mercury-insensitive water channel) has been detected in the gills of *Squalus acanthias*. Branchial mRNA expression was low relative to other tissues and curiously declined after acclimation to 120% seawater (Cutler et al., 2011a), but protein expression was detected by immunohistochemistry (IHC) in both the acid-excreting and base-excreting MRC morphotypes (Cutler et al., 2012). A study examining the diffusive water permeability of isolated gill cell membranes in *Squalus acanthias* concluded that the apical rather than the basolateral membrane was the limiting factor, but activation energy data were not indicative of the participation of aquaporins (Hill et al., 2004). Furthermore, permeability values were similar to those in gill cell membranes of a marine teleost. Clearly this is an area where our understanding is fragmentary and more work is needed.

2.2.2. KIDNEY

The anatomical complexity of nephrons in marine elasmobranchs (see Section 4.2.2) rivals that in mammals (Hentschel and Elger, 1989; Lacy and Reale, 1995; Evans et al., 2004). The key role of the kidney is conserving urea and TMAO (see Section 4.2.2) while excreting excess ions and metabolites. Functionally, the overall glomerular filtration rates (GFRs) and urine flow rates (UFRs) are high relative to those of marine teleosts. Measured GFRs range from 0.2–12 ml kg⁻¹ h⁻¹ (see Hickman and Trump, 1969 for a review of early measurements; Goldstein and Forster, 1971a; Forster et al., 1972; Schmidt-Nielsen et al., 1972; Brown and Green, 1987; Benyajati and Yakota, 1990; Wells et al., 2002; Janech et al., 2006b), while UFRs are typically 0.10–1.0 ml kg⁻¹ h⁻¹ (see Table 5.2). Increases in GFR in volume-loading situations such as salinity reduction probably reflect increases in the number of filtering nephrons and in their single nephron filtration rates (Goldstein and Forster, 1971a; Forster et al., 1972; Brown and Green, 1987; Wells et al., 2002; Janech et al., 2006b). The closure of glomerular bypass shunts may be involved (Brown and Green, 1992). Na⁺,

Cl^- , Ca^{2+} , urea, TMAO, and water are reabsorbed on a net basis from the primary filtrate, while toxic divalents (Mg^{2+} , SO_4^{2-}) and inorganic phosphate are secreted; as noted earlier, the excreted urine is never hyperosmotic to the blood plasma. Quantitatively, the contribution of the kidney to NaCl excretion is slightly less than that of the rectal gland, and much less than that of the gill (Table 5.1). However, when the rectal gland is removed, the kidney compensates by increasing urinary Cl^- excretion (and presumably Na^+ excretion) and this is achieved mainly by increasing UFR up to 5-fold, with unchanged or increased Cl^- concentrations (Burger, 1962, 1965; Chan et al., 1967).

Under normal conditions in full strength seawater, the renal tubules reabsorb Na^+ , Cl^- , urea, TMAO, and water from the filtrate with high efficiency (>80%), but this falls when the animals are volume-loaded or encounter more dilute environments (Forster et al., 1972; Schmidt-Nielsen et al., 1972; Wong and Chan, 1977; Wells et al., 2002; Janech et al., 2006b). Concentration gradients for Na^+ , Cl^- , and water have been detected at a macroscopic level between the dorsal and ventral parts of the kidney in *Raja erinacea* (Hentschel et al., 1986). In *Squalus acanthias*, Schmidt-Nielsen et al. (1972) presented convincing evidence for a tight, fixed coupling of Na^+ reabsorption to urea reabsorption (molar ratio = 1.6:1) against an uphill urea gradient. This is suggestive of Na^+ -linked active transport, similar to Na^+ -linked glucose reabsorption, which operates with similar stoichiometry in elasmobranch nephrons (Kipp et al., 1997; Althoff et al., 2006, 2007). To our knowledge, this concept of Na^+ -linked urea reabsorption has never been disproven, and indeed has been supported by brush-border membrane vesicle studies on *Raja erinacea* (Morgan et al., 2003b). Furthermore, some Na^+ -linked glucose transporters can also transport urea (Wright et al., 2004). However, Na^+ -linked urea transport would support an active model of urea transport, in conflict with the proposed passive counter-current multiplication mechanisms for urea reabsorption in the elasmobranch nephron (see Section 4.2.2.1).

NKA activity is relatively high in the kidney (Hayslett et al., 1973; Piermarini and Evans, 2000; Pillans et al., 2005; Dowd et al., 2010) and immuno-histochemical (IHC) studies have shown it to be widely distributed in most segments of the nephron, though notably absent from the collecting tubule of *Triakis scyllia* (Fig. 5.1) (Hyodo et al., 2004). Electrophysiological, pharmacological, micropuncture, and net flux studies on *in situ* tubules of *Raja erinacea* (Stolte et al., 1977) and perfused tubules of *Squalus acanthias* (Friedman and Hebert, 1990; Hebert and Friedman, 1990) have shown that Na^+ and Cl^- reabsorption occurs in the diluting segment of the nephron, a region effectively impermeable to water. This appears to be mediated via an absorptive apical Na^+ , K^+ , 2Cl^- cotransporter running off the Na^+

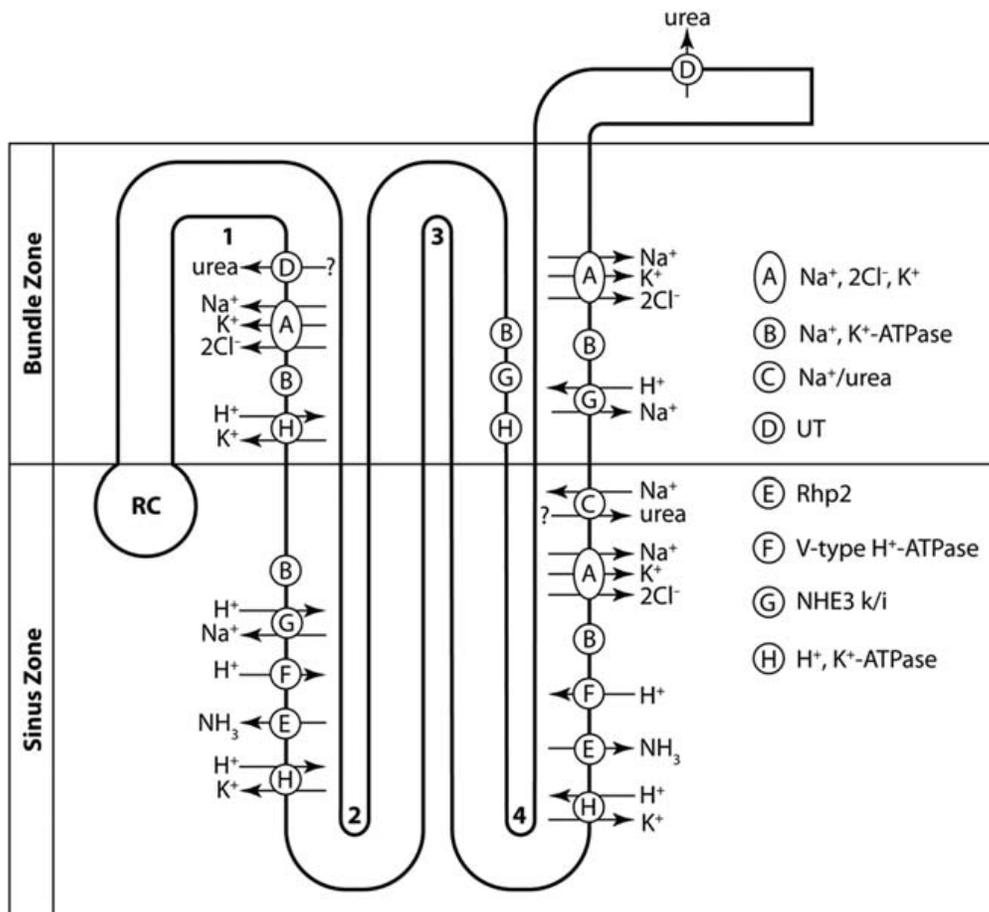


Figure 5.1. A schematic representation of a single nephron within the elasmobranch kidney. The renal corpuscle (RC) or glomerulus leads to two dorsal (Loops 1 and 3) tubule segments encased in a pertubular sheath (bundle zone) and there are two ventral (Loops 2 and 4) segments in the sinus zone. Filtrate moves from the RC through the 4 loops to the final distal tubule and collecting duct before excretion of urine to the environment. The tubule location of transporters was taken from various publications: Na^+ , 2Cl^- , K^+ cotransporter (Biemesderfer et al., 1996); Na^+ , K^+ -ATPase (NKA) (Li et al., 2013), Na^+ /urea antiporter (Morgan et al., 2003b), facilitated urea transporter (UT) (Morgan et al., 2003b; Hyodo et al., 2004); Rhesus glycoprotein, Rhp2 (Nakada et al., 2010); V-type H^+ -ATPase (Nakada et al., 2010); Na^+ , H^+ antiporter, NHE3k/i (Li et al., 2013); H^+ , K^+ -ATPase (Swenson et al., 1994). The “?” marks associated with urea transporters C (loop 1) and D (loop 4) refer to the uncertainty of exact tubule localization (see Morgan et al., 2003). The nephron structure was based on a schematic proposed by Lacy and Reale (1985).

electrochemical gradient established by basolateral NKA activity (Friedman and Hebert, 1990; Hebert and Friedman, 1990). The presence of apical Na^+ , K^+ , 2Cl^- cotransporter in this segment and other parts of the tubule (proximal, distal) was later confirmed by IHC (Biemesderfer et al., 1996).

Recently, in *Triakis scyllium*, an unusual isoform of the Na^+/H^+ exchanger (NHE3k/i) has been found in the apical membranes in the diluting segment, as well as in the proximal and early distal tubule (Li et al., 2013), so this may also contribute to Na^+ reabsorption. Biemesderfer et al. (1996) also found IHC evidence for a basolateral secretory Na^+ , K^+ , 2Cl^- cotransporter isoform in the proximal tubule, which may explain earlier electrophysiological evidence for Cl^- secretion in the proximal tubule (Fig. 5.1) (Beyenbach and Fromter, 1985). Localization of Cl^- , Na^+ , and Mg^{2+} in apical vesicles (Hentschel and Zierold, 1994), high Mg^{2+} , Ca^{2+} , SO_4^{2-} , and inorganic phosphate concentrations in tubular fluid in the proximal segment, particularly proximal tubular segment II (PTS II; Stolte et al., 1977), and studies on isolated sealed PTS II tubules directly demonstrating secretion of Na^+ , Cl^- , Mg^{2+} , and fluid (Sawyer and Beyenbach, 1985; Beyenbach, 1995) also suggest that the proximal tubule serves a secretory role. The mechanism of Mg^{2+} secretion remains unknown, but it may be regulated through a Mg^{2+} -sensing calcium receptor that has been identified in the kidney of *Squalus acanthias* (Hentschel et al., 2003). The final urine composition, and its changes during salinity variations, therefore will depend on the balance between secretory and absorptive processes for each ion, as well as the filtered load at the glomerulus. Acidification of the urine (see Section 3.2.2) may occur in both proximal and distal tubules based on H^+ , K^+ -ATPase, V-type H^+ -ATPase, NHE3k/I, and carbonic anhydrase distribution (Endo, 1984; Hentschel et al., 1993; Swenson et al., 1994; Nakada et al., 2010; Li et al., 2013).

2.2.3. RECTAL GLAND

Ever since the discovery by Burger and Hess (1960) that the rectal gland serves as a powerful NaCl secretory organ, this small digitiform organ in the elasmobranch colon has created excitement among transport physiologists. The gland is packed with MRCs rich in NKA activity, and specific activity is much higher than in gills or kidney (Morgan et al., 1997; Piermarini and Evans, 2000; Wilson et al., 2002; Pillans et al., 2005; Dowd et al., 2010). Its cellular and 3-dimensional tubular structure is relatively simple, unlike the gills and kidney (Oguri, 1964). The organ can easily be cannulated for perfusion and collection of the secretion product *in situ* or *in vitro*, its tubules are perfused without difficulty, and its cells can be cultured as flat epithelia *in vitro*. Literally 100s of papers have analyzed its transport and control mechanisms. This literature has been extensively reviewed (e.g., Greger et al., 1986; Shuttleworth, 1988; Riordon et al., 1994; Silva et al., 1996, 1997; Hazon et al., 1997; Olson, 1999), most recently by Epstein and Silva (2005), the latter highlighting discoveries on *Squalus acanthias* that appeared in the Mount Desert Island Biological Laboratory (MDIBL) Bulletin. The reader

is referred to these resources, as well as Chapter 4, for details on the secretory pathway and its neuroendocrine controls.

Suffice it to say that the secretory mechanism for NaCl is the classic “Silva” model of “secondary active transport” (Silva et al., 1977a,b) featuring a basolateral secretory Na^+ , K^+ , 2Cl^- cotransporter for Na^+ and Cl^- entry running off the Na^+ electrochemical gradient established by basolateral NKA, with apical transcellular exit of Cl^- by a CFTR-like channel and paracellular extrusion of Na^+ . The secretion is approximately isosmotic to the blood plasma (i.e., close to $1000 \text{ mOsmol L}^{-1}$) and consists of an almost pure 500 mmol L^{-1} NaCl solution; the composition is essentially independent of production rate. Water moves passively with NaCl transport, and virtually all other substances are excluded, most notably urea ($<20 \text{ mmol L}^{-1}$ in *Squalus acanthias* – Burger and Hess, 1960; Zeidel et al., 2005; $\sim 80 \text{ mmol L}^{-1}$ in *Hemiscyllium plagiosum* – Wong and Chan, 1977). Aquaporins may be involved in water permeation because AQP4 is abundantly expressed in the rectal gland of *Squalus acanthias* (Cutler et al., 2011a, 2012); however the biophysical properties of the rectal gland cell membranes were not indicative of aquaporin involvement (Zeidel et al., 2005). Of the other ions measured in the secretion (Ca^{2+} , Mg^{2+} , SO_4^{2-} , phosphate all $<1 \text{ mmol L}^{-1}$), only K^+ is present at close to plasma levels ($\sim 5 \text{ mmol L}^{-1}$; Burger and Hess, 1960; Chan et al., 1967; Wood et al., 2007c). In *Squalus acanthias*, activation of secretion occurs by two polypeptides: vasoactive intestinal polypeptide (VIP) released from nerves acting as an endogenous neurotransmitter and C-type natriuretic peptide (CNP) released from the heart acting as a hormone. In *Scyliorhinus canicula*, scyliorhinin II (originally known as rectin – Shuttleworth and Thorndyke, 1984; Anderson et al., 1995) seems to play a similar role to VIP, and the latter is ineffective in this species (Shuttleworth, 1983) as well as in *Leucoraja erinacea* (Kelley et al., 2014). Anderson (Chapter 4) gives a more complete overview of control mechanisms.

Our focus here is on the osmoregulatory role of the rectal gland in the whole organism. In freely swimming animals, rectal gland flow is intermittent and irregular (Burger and Hess, 1960; Burger, 1962, 1965), and almost nonexistent in quietly resting, fasting animals (Wood et al., 2007c). Averaged over prolonged periods, rectal gland NaCl excretion appears to be greater than that through the kidney, but less than that through the gills (Table 5.1). As noted earlier, elasmobranchs can maintain ionic homeostasis when the rectal gland is ligated or removed (see Section 2.2.1), but they deal less effectively with exogenous NaCl loads, which normally activate increased rectal gland secretion (Burger, 1965; Chan et al., 1967; Haywood, 1975a). Herein is the key role of the rectal gland, to deal with the extra NaCl and volume loads accompanying feeding on salty food (e.g., invertebrates)

and/or the ingestion of seawater during feeding. Indeed infusion of a 500 mmol L⁻¹ NaCl solution for 6 h resulted in a brisk rise in the excretion of a 500 mmol L⁻¹ NaCl solution via the rectal gland, amounting to >70% of the infused load over 24 h (Wood et al., 2007c). Based on effects seen with various injected solutions in intact *Squalus acanthias*, Burger (1962) speculated that there were 3 separate stimuli – volume, osmotic, and NaCl. Classic physiological experiments using dogfish that were artificially-ventilated and pithed or anesthetized established a primary role for volume expansion rather than NaCl or tonicity; furthermore, by an elegant cross-perfusion technique, these studies proved that a blood-borne agent served as the mediator (Solomon et al. 1984a,b, 1985; Erij and Rubio, 1986). This is now known to be CNP, which is released from the heart in response to volume expansion (probably sensed as central venous pressure – Erij and Rubio, 1986). CNP acts both directly and indirectly (by mobilizing VIP) to activate secretion by the gland.

In elasmobranchs living in full strength seawater, feeding and/or accompanying ingestion of seawater are probably the normal causes of rectal gland activation. Dietary NaCl loading markedly increased the activity of NKA in the rectal gland of *Scyliorhinus canicula*, apparently by a post-translational mechanism because mRNA increases lagged behind (MacKenzie et al., 2002). NaCl and NaHCO₃ infusions had a similar effect on rectal gland NKA activity in *Squalus acanthias* (Wood et al., 2008). A recent suite of studies on *Squalus acanthias* has shown that after a large meal, there are marked increases (with peaks at 6–20 h post-feeding) in aerobic and anaerobic metabolic enzyme activities as well as NKA activity in the gland (Walsh et al., 2006), changes in tubular and cellular structure symptomatic of increased secretory activity (Matey et al., 2009), and both proteomic (Dowd et al., 2008) and transcriptomic changes (Deck et al., 2013) indicative of some of these responses. The post-prandial “alkaline tide” (see Sections 3.2.2 and 3.2.4) may help promote these phenomena (Wood et al., 2008) because secretion is augmented by metabolic alkalosis both *in vitro* (Shuttleworth et al., 2006) and *in vivo* (Wood et al., 2007c). However, as yet there are no direct measurements of rectal gland secretion after feeding. Bucking (Chapter 6) provides additional information.

The other normal cause of volume loading would be environmental dilution. Increased rectal gland secretion was shown in the original experiments of Burger (1965) when *Squalus acanthias* were exposed to 72–82% seawater, and this was confirmed in *Hemiscyllium plagiosum* exposed to 50% seawater (Wong and Chan, 1977). Much later, *in vitro* experiments with perfused glands of *Scyliorhinus canicula* demonstrated increased secretion in glands from *Scyliorhinus canicula* exposed to 70% seawater and decreased secretion in those from animals exposed

to 120% seawater (Anderson et al., 2002a). Even though the animals are probably taking up less NaCl at the gills in dilute environments, they are taking up more water, so the response is homeostatic in excreting more water and dumping Na⁺ and Cl⁻ so as to help reestablish osmotic equilibrium. In this respect, the rectal gland response is complementary to that of the kidney. Nevertheless, Na⁺ and Cl⁻ concentrations in the rectal gland secretion are reduced, so overall excretion rates fall.

2.2.4. GUT

The unusual functional anatomy of the gastrointestinal tract of elasmobranchs has been reviewed by Bucking (Chapter 6). Historically, the gut has received the least attention of the four osmo/ionoregulatory sites, probably because Homer Smith (1931b, 1936) concluded that marine elasmobranchs had no need to drink the external medium. However, careful measurements with modern techniques (reviewed by Anderson et al., 2007) have shown that drinking does occur, albeit at a very low rate (generally < .5 ml kg⁻¹ h⁻¹; Table 5.2), which is about 10% of that in marine teleosts. This is probably insufficient to supply the fluid requirements of the urinary and rectal gland flows. Furthermore, there is no absolute proof that the ingested water or ions from drinking are actually absorbed *in vivo*, though ²²Na⁺ infused into the intestine of intact *Scyliorhinus canicula* appeared in the bloodstream within 1 h (Hazon et al., 1997) and aquaporin AQP4 is expressed throughout the tract (Theodosiou and Simeone, 2012; Cutler et al., 2011a, 2012).

Drinking is under the control of the renin-angiotensin system, and decreases when the animal is challenged with lower salinity, and *vice versa* (Hazon et al., 1989, 1997; Anderson et al., 2002b). CNP appears to inhibit drinking induced by angiotensin II (Anderson et al., 2001; reviewed by Anderson, Chapter 4). As with the rectal gland, changes in volume, probably acting through changes in blood pressure, appear to be key stimuli, though in a reciprocal fashion; acute volume increments elevate the secretion rate of the rectal gland and depress drinking rate, so the responses are complementary. A decrease in blood volume of only 6% was sufficient to initiate a 36-fold increase in drinking in *Scyliorhinus canicula* (Anderson et al., 2002b)!

Perhaps of greater importance, but often overlooked, are the water and ions obtained from the food (especially from hypotonic teleost prey or salty invertebrate prey), and from seawater that may be “accidentally” ingested along with the food (reviewed by Wood and Bucking, 2011). For example, the osmoregulatory benefits for a marine elasmobranch (internal osmolality ~1050 mOsmol L⁻¹) to eat a marine teleost (internal osmolality ~350 mOsmol L⁻¹) are obvious. Recently, the “fate” of ions and water in the digestive tract chyme has been addressed in several *in vitro* studies

(see Chapter 8; Anderson et al., 2007, 2010, 2012, 2015; Theodosiou and Simeone, 2012; Liew et al., 2013) and two *in vivo* investigations (Wood et al., 2007b, 2009). The first *in vitro* studies, on intestinal sac preparations from fasted *Chilloscyllum plagiosum* (Anderson et al., 2007, 2010) and fasted *Leucoraja erinacea* (Anderson et al., 2010; Theodosiou and Simeone, 2012) and *Raja egalinteria* (Anderson et al., 2010) showed net secretion of urea into the mucosal or “chyme” solution, as well as net absorption of Na^+ , Cl^- , and water, the latter at a rate comparable to routine drinking rates in other species (Table 5.2).

In an *in vivo* study, *Squalus acanthias* were sacrificed for chyme analysis at various times after a large voluntary meal of teleost fish (5.5% body weight), which took more than 5 days to fully digest and absorb (Wood et al., 2007b). Interestingly, despite the hypotonic meal, chyme osmolality in all compartments remained equal to that of blood plasma at all times, so it was rapidly adjusted by the addition of osmolytes. Initially, this occurred by the apparent secretion of urea, Na^+ , and especially Cl^- in the cardiac and pyloric stomach compartments. Seawater drinking, though not measured directly, appeared to continue at only a low rate during digestion, based on the analysis of chyme ions. While there was an early addition of fluid in the two sections of the stomach, likely associated with gastric HCl secretion (MacIntosh, 1936), most of the original water content of the prey was eventually absorbed in the intestine (spiral valve) on a net basis. In a subsequent study on the alkaline tide phenomenon (see Section 3.2.4), the increase in Cl^- concentration of the gastric chyme was slowed by omeprazole, a blocker of gastric H^+ , K^+ -ATPase (Wood et al., 2009). In the intestine, the high NaCl content of the chyme was partially replaced by a substantial further addition of urea of uncertain origin (biliary, pancreatic, or intestinal wall secretion – see Section 4.2.4). Eventually, urea, Na^+ , K^+ , Cl^- , and water were all largely absorbed or reabsorbed across the intestine, while Mg^{2+} and Ca^{2+} were largely excluded.

In vitro gut sac (Liew et al., 2013) and isolated intestinal epithelial studies using Ussing chambers (see Chapter 8; and Anderson et al., 2012, 2015), both set up with identical physiological salines on the two sides, have clarified some of these patterns in *Squalus acanthias*. For example, net Cl^- secretion occurs mainly in the pyloric stomach, and there is strong net Na^+ , Cl^- , water, and urea reabsorption across the intestine after feeding. The colon contributes to final water and urea reabsorption (see also Theodosiou and Simeone, 2012). Furthermore, under fasting conditions, the intestine secretes urea while all the other sections of the tract absorb urea at a low rate, but after feeding, this changes over to a high rate of urea uptake, but only across the wall of the intestine. This involves an apparent active, phloretin-sensitive, Na^+ -coupled process (see Section 4.2.4). However, there

remain some uncertainties and discrepancies between the *in vivo* and *in vitro* studies. For example, in the latter, all sections of the tract, especially the intestine, absorbed fluid and secreted K^+ in both fed and fasted preparations, so there was no evidence of the apparent fluid secretion in the stomach segments, or of K^+ reabsorption in the intestine, seen *in vivo*. Curiously, phloretin promoted net Mg^{2+} and Ca^{2+} secretion by the intestine, while having no effect on net Na^+ , Cl^- , and water reabsorption. Recently an unusual Na^+/H^+ exchanger (NHE3k/i) has been identified in the apical membranes of enterocytes of the intestine and rectum of *Triakis scillium* (Li et al., 2013). Essentially nothing is known about the transport mechanism(s) for any ion in the elasmobranch intestine; this is a rich area for future investigation.

2.3. Freshwater and Intermediate Salinities

Virtually all marine elasmobranchs can tolerate some dilution of the external medium (e.g., down to $\sim 70\%$ seawater, ~ 23 ppt) and about 20% are routinely recorded in dilute environments in nature (Martin, 2005). Most of these are “tolerators,” and about half (i.e., $\sim 10\%$) of these tolerators reside for long periods in the brackish waters of estuaries. Only a very few elasmobranch species ($\sim 1\%$) such as the bull shark (*Carcharhinus leucas*), the sawfish (*Pristis microdon*), and the Atlantic stingray (*Dasyatis sabina*) are truly euryhaline (i.e., able to live in seawater, freshwater, and all intermediate salinities) while a single Family of 20–30 species (2–3%), the Potomotrygonidae stingrays of South America, are stenohaline freshwater animals, intolerant of saline waters. Two excellent and exhaustive reviews, including one in a recent volume of *Fish Physiology* have summarized the iono- and osmoregulatory mechanisms employed by elasmobranchs in each of these groups (Ballantyne and Robinson, 2010; Ballantyne and Fraser, 2013), so it would be redundant to repeat this material here. Instead, we will briefly summarize the key strategies of the “tolerators,” and estuary inhabitants (which are generally similar), the facultative (euryhaline) freshwater elasmobranchs and the obligate (stenohaline) freshwater elasmobranchs. The major difference between the two freshwater groups is that the facultative group has maintained the capacities to synthesize and retain high urea levels in their body fluids, thereby incurring osmotic costs but retaining the ability to osmoregulate in seawater, while the obligate group has irreversibly lost these capacities. The facultative group also has a much shorter evolutionary history in freshwater (a few million years) than the obligate group (20–100 million years).

2.3.1. DILUTION TOLERATORS

Representative studies include Chan and Wong (1977a,b), Wong and Chan (1977), Goldstein and Forster (1971a), Schmidt-Nielsen et al. (1972), Payan et al. (1973), Wells et al., (2002), Sulikowski et al. (2003), Cooper and Morris (1998, 2004), Dowd et al. (2010), Wosnick and Freire (2013), and Guffey and Goss (2014). As tolerators encounter more dilute environments, their basic strategy is to lower their osmolality as much as tolerable so as to minimize the osmotic gradient for water entry and consequent volume expansion and weight gain. This is achieved by decreasing urea and TMAO levels, and to a lesser extent by decreasing Na^+ and Cl^- concentrations in the extracellular fluid. Much of the decline in these concentrations is due to the gain of water and accompanying dilution, rather than a large loss of ion content from the body. There is some indication that plasma $[\text{Cl}^-]$ is retained more effectively than plasma $[\text{Na}^+]$, perhaps because the dilution-induced alkalosis activates the Cl^- uptake *versus* base excretion mechanism on the gills (see Section 2.2.1). Plasma $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ also decline, but appear to be better regulated than $[\text{Na}^+]$ and $[\text{Cl}^-]$. Gill loss rates of ions decrease as the gradients decline, but the role of the gills in overall ion balance during dilution remains unclear. The ultimate limiting feature of low salinity tolerance may be an inability of the gills to activate net ion uptake in dilute media. Urea, TMAO, free amino acids, and ions are also lost from the intracellular compartment. At the same time, drinking decreases or stops, rectal gland secretion rate and urinary output rate both increase greatly, which promotes greater water and ion excretion, with the renal response additionally promoting urea and TMAO losses. GFR increases markedly, and the balance between tubular secretion and tubular reabsorption of water and NaCl shifts away from the latter, while urea and TMAO reabsorption decrease. Urea production rate by the OUC may also slow down in some species. Urea loss across the gills generally decreases in proportion to the decreased outward concentration gradient. As the normal motivation for entering dilute environments is feeding opportunity, ions gained from the food likely become more important in the overall electrolyte economy. Information on how long, if ever, it takes for these adjustments to reach a new steady state in tolerators is variable, with a range from 4 d to never, depending on the species and the extent of environmental dilution.

2.3.2. FACULTATIVE FRESHWATER ELASMOBRANCHS

Representative studies include Smith (1931a), Thorson (1962, 1967), Thorson et al. (1973), De Vlaming and Sage (1973), Gerzeli et al. (1976), Piermarini and Evans (1998, 2000, 2001), Piermarini et al. (2002), Janech and Piermarini (2002), Pillans and Franklin (2004), Choe et al. (2004, 2005),

Pillans et al. (2005, 2006, 2008), Janech et al. (2006b), and Reilly et al. (2011). The strategy of these truly euryhaline animals in freshwater is in fact the ultimate perfection of that shown less effectively by the dilution tolerators, and all the same qualitative changes seem to occur as these elasmobranchs enter dilute environments. The difference however is that although ions, urea, and TMAO initially decline in the body fluids, internal osmolality eventually stabilizes at $\sim 550\text{--}650\text{ mOsmol L}^{-1}$, at which point no further ions or N-osmolytes are lost on a net basis. This stabilization is facilitated by two additional compensations that are not seen in the tolerators: (i) an ability to actively take up ions, particularly Na^+ and Cl^- , from the very dilute external environment at the gills, and (ii) an ability to strongly reabsorb ions at the kidney despite very high rates of water entry, GFR, and UFR. The former is presumptive and has never been directly demonstrated to our knowledge. However, greatly increased branchial NKA activities or mRNA levels in some species, higher mRNA levels and/or IHC staining for other freshwater ion uptake proteins (e.g., NHEs), and a proliferation of MRC distribution onto the respiratory lamellae in freshwater acclimated specimens all provide a strong indication that this occurs. High GFR, UFR, and low ion concentrations in the urine comparable to those of freshwater teleosts have been directly demonstrated in several species, as well as greatly increased kidney NKA activity. Gut NKA activity does not change markedly. In some but not all euryhaline species, the rectal gland shrinks, and its NKA activity declines with freshwater acclimation, suggesting a decline in secretory activity. As a result, these elasmobranchs can live indefinitely in freshwater despite retaining still elevated levels of urea and TMAO in the body fluids, at least relative to teleosts. Nevertheless, they retain the capacity to reverse all these changes, including the reduction of urea production by the OUC, when returning to higher salinities.

2.3.3. OBLIGATE FRESHWATER ELASMOBRANCHS

Traditionally, this category comprises only the Potomotrygonid stingrays, many of which thrive in the ion-poor acidic “blackwater” rivers of South America. These animals are characterized by an intolerance of elevated salinities much above 50% seawater (~ 17 ppt), and an inability (or minimal ability) to make urea by the OUC, to retain it at the kidney, or to elevate it in the face of salinity challenge. However, an interesting “intermediate” may be *Himantura signifer*, a freshwater stingray of Southeast Asia that tolerates only up to $\sim 60\%$ seawater (~ 20 ppt), but still synthesizes urea (see Section 4.3.2).

With respect to the Potomotrygonid stingrays, representative studies include Thorson et al. (1967, 1978), Thorson (1970), Griffith et al. (1973), Carrier and Evans (1973), Gerst and Thorson (1977), Bittner and Lang (1980), Wood et al. (2002, 2003), and Duncan et al. (2009, 2010, 2011).

Typically, these freshwater elasmobranchs maintain plasma Na^+ , Cl^- , divalent ions, urea, TMAO, and osmolality all at levels similar to those of freshwater teleosts. A small rectal gland is present but appears to be atrophied and nonfunctional, a relic of the marine origin. Radiotracer investigations with $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ have shown apparent active, carrier-mediated Na^+ and Cl^- uptake across the gills and/or body surface. These processes exhibit saturation kinetics and a similar sensitivity to pharmacological inhibitors as in freshwater teleosts. High levels of dissolved organic carbon (DOC), which is the cause of the tea color of the “blackwaters,” reduce efflux rates and promote influx rates of Na^+ and Cl^- at low pH. Nevertheless, net balance remains negative, suggesting that ions from food must constitute an important part of the normal electrolyte economy. Gill and kidney NKA activities are higher in specimens collected from ion-poor, acidic “blackwater” rivers than in those from more ion-rich, circumneutral, “whitewaters.” MRCs occur on the respiratory lamellae as well as the gill filaments, as in freshwater teleosts, and are particularly abundant on the 4th gill arch. We are aware of no information on drinking, gut function in ionoregulation, water turnover rates, or kidney function in these unique fish. These areas, together with the interesting protective actions of DOC on ionoregulation, comprise a wide arena for future investigations.

3. ACID–BASE BALANCE

Previous reviews include [Murdaugh and Robin \(1967\)](#), [Heisler \(1988\)](#), [Evans et al. \(2004\)](#), and [Ballantyne and Fraser \(2013\)](#). Respiratory aspects of acid–base physiology are discussed by Morrison et al. in Chapter 3.

3.1. Overview

Without doubt, the gills are the most important organ of acid–base balance in elasmobranchs, accounting for virtually all of the exchange of acid–base equivalents with the water. Their ability to regulate acid–base status by “respiratory” changes in ventilation or perfusion appears to be quite limited, but certainly they are sites at which “respiratory” disturbances in the environment (e.g., low or high PO_2 , high PCO_2 , high ammonia) can be transmitted to the animal, causing disturbances in internal acid–base status (see Chapter 3). Acid–base fluxes are small at the kidney, nonexistent at the rectal gland, but quite large in the gastrointestinal tract, and are associated with the digestion of food. It is unclear whether the latter are transmitted directly to the environment via the gut, but certainly they are transmitted to

the internal environment of the animal, and then to the external environment through the gills.

3.2. Seawater

3.2.1. GILLS

It has long been known that the gills have a spectacular ability to excrete acid or base loads to the external environment, with flux rates up to $1000 \mu\text{mol kg}^{-1} \text{h}^{-1}$. Acid excretion or base uptake can be induced by “external” hypercapnia associated with high environmental PCO_2 , or “internal” hypercapnia induced by the CO_2 retention that accompanies environmental hyperoxia; a compensatory accumulation of HCO_3^- in the blood plasma brings the arterial blood pH, pH_a , back close to normal within 6–24 h (Cross et al., 1969; Randall et al., 1976; Heisler et al., 1975, 1988; Evans et al., 1982; Graham et al., 1990; Wood et al., 1990; Claiborne and Evans, 1992; Choe and Evans, 2003). Branchial acid excretion can also be induced by the direct infusion of HCl (Tresguerres et al., 2005) or acidifying salts such as NH_4Cl (Wood et al., 1995). Base loads, induced either by NaHCO_3 infusion (Hodler et al., 1955; Swenson and Maren, 1987; Claiborne and Evans, 1992; Wood et al., 1995; Tresguerres et al., 2005, 2006) or by the “alkaline tide” accompanying feeding (Wood et al., 2005, 2007a,b, 2009; Tresguerres et al., 2007; see Section 3.2.4) can be excreted with equal speed.

The physiological evidence that acid excretion is linked to Na^+ uptake is stronger than the evidence that base excretion is linked to Cl^- uptake at the gills. In *Scyliorhinus canicula*, injections of HCl and various NH_4^+ salts caused increases in unidirectional influxes and effluxes of Na^+ and the net flux became more positive (Payan and Maetz, 1973). Na^+ influx increased as blood pH decreased. Both Na^+ influx and Cl^- influx were dependent on external Na^+ and Cl^- concentrations, the former in a clear Michaelis–Menten fashion (Bentley et al., 1976). During exposure to elevated PCO_2 , various marine elasmobranchs excreted acid and/or took up base from the external seawater, resulting in HCO_3^- accumulation in the blood plasma (Randall et al., 1976; Heisler et al., 1975, 1988; Evans et al., 1982; Claiborne and Evans, 1992; Choe and Evans, 2003). In only one of these studies was there a clear increase in total ammonia excretion as well (*Squalus acanthias*; Claiborne and Evans, 1992). The net acid excretion was dependent on the presence of external Na^+ (Evans et al., 1982) and in the euryhaline stingray, *Dasyatis sabina*, was faster in seawater (high $[\text{NaCl}]$) than in freshwater (low $[\text{NaCl}]$) (Choe and Evans, 2003). Many studies have demonstrated greatly increased base excretion through the gills in response to metabolic alkalosis caused by experimental HCO_3^- loading (Hodler et al., 1955; Claiborne and

Evans, 1992; Wood et al., 1995; Tresguerres et al., 2005, 2006) or feeding (“alkaline tide”; Wood et al., 2007a, 2009; Tresguerres et al., 2007), but the linkage to Cl^- uptake has yet to be clearly demonstrated, with no Cl^- flux measurements and only some of the studies detecting a fall in plasma $[\text{Cl}^-]$. There are also no clear data on whether plasma $[\text{Na}^+]$ changes during the compensation of an acid–base disturbance, but the expected changes (a few mM) would be very difficult to detect against the high background $[\text{Na}^+]$ in elasmobranch plasma. Overall, these data suggest that Na^+/H^+ and perhaps $\text{Na}^+/\text{NH}_4^+$ and $\text{Cl}^-/\text{HCO}_3^-$ exchanges occur at the gills for acid–base regulation, as in freshwater and marine teleosts, and increase the animal’s electrolyte load at times of disturbance.

Modern molecular evidence, relying heavily on IHC, has identified two types of MRC involved in acid–base fluxes. Fig. 5.2 illustrates current ideas about the functions of these two MRC types, while Fig. 3.13 of Wegner (2015) illustrates a false color IHC image of these two cell types in *Triakis*

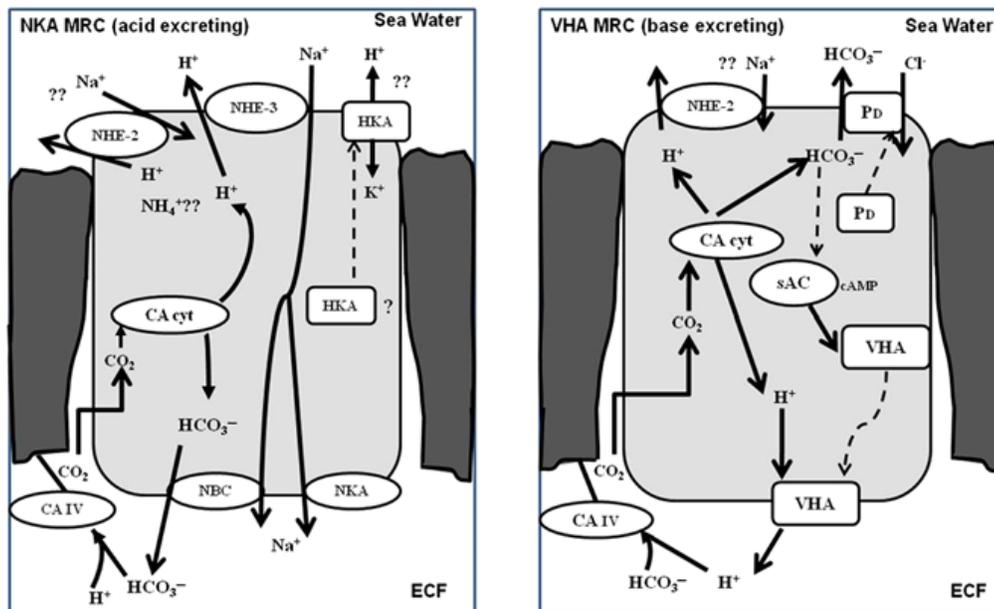


Figure 5.2. Models summarizing current ideas about the function and spatial distribution of transporters and enzymes important in acid–base balance in two MRC types, the putative acid-excreting cells (“NKA cells”) and putative base-excreting cells (“VHA cells”), in the gills of marine elasmobranchs. Dashed lines indicate the migration of transporters from the Golgi membranes or intracellular vesicles to the apical or basolateral membranes. NKA, Na^+ , K^+ -ATPase; VHA, V-type H^+ -ATPase; CA cyt, cytoplasmic carbonic anhydrase; CA IV, extracellular membrane-bound carbonic anhydrase; NBC, sodium bicarbonate cotransporter; NHE-2, sodium-hydrogen exchanger 2; NHE-3, sodium-hydrogen exchanger 3; P_D , pendrin (chloride-bicarbonate exchanger); HKA, H^+ , K^+ -ATPase; sAC, soluble adenylyl cyclase; cAMP, cyclic adenosine monophosphate; ??, entirely speculative. See text for further details.

semifasciata. Both of these reside mainly on the gill filaments in the interlamellar region, with few on the lamellae in seawater-acclimated fish. Both also contain abundant intracellular carbonic anhydrase (Wilson et al., 2000; Tresguerres et al., 2007), not to be confused with the extracellular carbonic anhydrase IV (Gilmour et al., 1997, 2001, 2007), which plays a key role in respiratory CO₂ excretion (see Chapter 3). One of these MRC types exhibits prominent protein expression of V-type H⁺-ATPase (“VHA cells”), and the other prominent protein expression of NKA (“NKA cells”), with some evidence for low numbers of intermediate cell types (Piermarini and Evans, 2001; Tresguerres et al., 2005; Reilly et al., 2011; Roa et al., 2014). The VHA cells express the anion exchanger pendrin in their apical membranes (Piermarini et al., 2002; Evans et al., 2004; Roa et al., 2014) while the NKA cells express the cation exchanger NHE3 (Choe et al., 2005, 2007) leading to a current model whereby VHA cells excrete base by Cl⁻/HCO₃⁻ exchange, energized by basolateral V-type H⁺-ATPase, and NKA cells excrete acid via Na⁺/H⁺ and/or Na⁺/NH₄⁺ exchange energized by basolateral NKA. Note that this is very different from the pattern in teleosts, where V-type H⁺-ATPase operates on the apical membrane for acid excretion.

The distribution of V-type H⁺-ATPase in VHA cells was originally described in *Squalus acanthias* by J. Wilson et al. (2002) as a “low level general diffuse pattern with an intense granular apical signal.” However, later studies by Piermarini and Evans (2001) in *Dasyatis sabina*, Tresguerres et al. (2005, 2006, 2007, 2010) in *Squalus acanthias*, and Roa et al. (2014) in *Triakis semifasciata* demonstrated that the distribution was mainly cytoplasmic under control and acidotic conditions, but became strongly localized to the basolateral membrane under alkalotic conditions (Fig. 5.2). Overall V-type H⁺-ATPase protein content of the cell membranes and V-type H⁺-ATPase enzymatic activity increased during experimental alkalosis or the post-feeding “alkaline tide” (Tresguerres et al., 2005, 2007). This translocation of the molecules from cytoplasmic vesicles to the basolateral membrane was inhibited by colchicine, a blocker of cytoskeleton-dependent cellular trafficking, and the acid-base disturbance in response to NaHCO₃ infusion was enhanced (Tresguerres et al., 2006). Selective blockade of gill carbonic anhydrase strongly reduced the ability of *Squalus acanthias* to excrete a HCO₃⁻ load (Swenson and Maren, 1987), and this also blocked the translocation of V-type H⁺-ATPase to the basolateral membrane and exacerbated the accompanying blood metabolic alkalosis (Tresguerres et al., 2007).

These observations led to the suggestion that intracellular H⁺ or HCO₃⁻ produced by intracellular carbonic anhydrase acts on a cytoplasmic “sensor” molecule, soluble adenylyl cyclase (sAC) to produce cAMP, thereby

activating the V-type H^+ -ATPase translocation (Fig. 5.2), mediated via the actin cytoskeleton (Tresguerres et al., 2007). Support for this idea was provided by biochemical and molecular identification of a sAC ortholog in dogfish gills, and demonstration of its Michaelis-Menten sensitivity to HCO_3^- concentrations within the physiological range (Tresguerres et al., 2010). Treatment with pharmacological blockers of sAC resulted in similar effects as seen previously with colchicine and carbonic anhydrase blockade: exacerbation of experimental metabolic alkalosis and inhibition of V-type H^+ -ATPase translocation to the basolateral membranes of VHA cells. The potential signaling cascade whereby extracellular alkalosis results in basolateral H^+ secretion and apical HCO_3^- extrusion has been described by Tresguerres et al. (2014). Interestingly, in *Squalus acanthias*, the branchial mRNA expression of V-type H^+ -ATPase was down-regulated in response to both high environmental ammonia exposure and infusions of ammonium salts (NH_4Cl , NH_4HCO_3), independent of acid–base status, suggesting that this transporter plays an additional role in modulating trans-gill ammonia fluxes (Nawata et al., 2015a,b).

Pendrin was first identified in *Dasyatis sabina* (Piermarini et al., 2002), and then in *Squalus acanthias* (Evans et al., 2004) and *Carcharhinus leucas* (Reilly et al., 2011) with a mainly subapical distribution in the VHA cells only. A recent report (Roa et al., 2014) indicates that it may move to the apical membrane during postprandial alkalosis in *Triakis semifasciata* in a similar manner to the basolaterally oriented translocation of V-type H^+ -ATPase (Fig. 5.2).

NHE-2 like and NHE-3-like immunoreactivities were first identified using heterologous antibodies in the gills of a range of elasmobranchs by Edwards et al. (2002) and Choe et al. (2002) and appeared to be associated with NKA cells. Later work with elasmobranch-specific antibodies demonstrated that NHE-3 localized in the apical membranes of NKA cells only (Choe et al., 2005, 2007; Reilly et al., 2011) (Fig. 5.2). However, the distribution of NHE-2 as assayed with a homologous antibody is less well defined; in *Squalus acanthias*, Claiborne et al. (2008) reported its presence in both NKA and VHA cells. Interpretation is further confounded by reports that NHE-2 expression at the mRNA level and/or protein level increased during experimental acidosis in some studies (Tresguerres et al., 2005; Nawata et al., 2015b), but not others (Choe et al., 2005; Claiborne et al., 2008). Furthermore, NHE3 did not respond to acidosis at either level (Choe et al., 2005, 2007) while NKA expression did increase (Tresguerres et al., 2005).

Another uncertainty is whether acid excretion occurs only as H^+ , or whether NH_4^+ excretion also contributes. In a recent investigation on *Squalus acanthias*, metabolic acidosis caused by HCl infusion did not cause any increase in ammonia excretion (Nawata et al., 2015b) in contrast to two

earlier studies on respiratory acidosis (hypercapnia) in this same species (Evans, 1982; Claiborne and Evans, 1992), where elevated ammonia excretion, presumably as $\text{Na}^+/\text{NH}_4^+$ exchange (Payan and Maetz, 1973), seemed to play a role in acid-base compensation. However, see Evans and More (1988) for opposing evidence. Furthermore, in *Dasyatis sabina*, there was no increase in ammonia excretion during the compensation of respiratory acidosis in seawater (Choe and Evans, 2003). This suggests that ammonia excretion is not involved in acid excretion, in agreement with earlier data showing that exposure to Na^+ -free seawater or amiloride did not affect ammonia excretion but greatly reduced acid excretion in this species (Evans et al., 1979).

Another potential acid excretion mechanism is H^+ versus K^+ exchange, and an H^+ , K^+ -ATPase, similar to that in the stomach (Smolka et al., 1994), has been identified in the gills of *Dasyatis sabina* (Choe et al., 2004) and *Squalus acanthias* (Evans et al., 2004). This enzyme is also restricted to NKA cells, but has a diffuse cytoplasmic distribution with no evidence of apical localization (Fig. 5.2). Furthermore its mRNA expression did not change in response to respiratory acidosis (Choe et al., 2004). Clearly there is a need for more work to understand the relative roles of NHE-2 versus NHE-3 versus H^+ , K^+ -ATPase, both in the NKA cells and elsewhere, as well as H^+ versus NH_4^+ excretion in branchial acid-base regulation in marine elasmobranchs.

3.2.2. KIDNEY

The kidneys of marine elasmobranchs do not make a large contribution to dynamic whole-body acid-base balance, but play an important role in HCO_3^- reabsorption. Renal mechanisms have been uncovered in this ancient group that have been retained by other vertebrate groups; therefore, there is an evolutionary importance to understanding the similarities and differences. Urine flow rates (Table 5.2) tend to be relatively high in marine elasmobranchs relative to marine teleosts (see Table 4.7 in Ballantyne and Fraser, 2013). Urine is acidic and pH is nearly constant in marine elasmobranchs (\sim pH 5.8; Dantzler, 1989). Acidification of the urine may occur in both proximal and distal tubules based on H^+ , K^+ -ATPase and carbonic anhydrase distribution (Endo, 1984; Hentschel et al., 1993; Swenson et al., 1994). Significant H^+ secretion and HCO_3^- reabsorption occur early in the nephron, in the proximal tubule (Dantzler, 1989). Tubular carbonic anhydrase plays an important role in acid secretion in the mammalian kidney (Pitts and Alexander, 1945), but appears to be much less important in dogfish shark. Surprisingly, when plasma HCO_3^- was elevated 3- to 7-fold in *Squalus acanthias*, there was little change in urine pH (Hodler et al., 1955; Swenson and Maren, 1986). Under these conditions, filtered

HCO_3^- was mostly reabsorbed and acid secretion was not impaired, even when carbonic anhydrase inhibitors were injected intravenously. Thus, these authors concluded that carbonic anhydrase was not involved in renal HCO_3^- reabsorption in dogfish.

H^+ secretion may involve several renal transporters. As in the mammalian kidney, brush border membrane vesicles of proximal tubules in *Squalus acanthias* display all the characteristics of an electroneutral Na^+/H^+ exchanger (Bevan et al., 1989). A gill NHE2 isolated in *Squalus acanthias* also shows mRNA expression in the kidney (Claiborne et al., 2008). Li et al. (2013) characterized a renal NHE3 in *Triakis scyllium* that shared more homology to mammalian than teleost NHE3 proteins. The NHE3 was localized to apical membranes of proximal tubules and distal tubules in *Triakis scyllium* (Fig. 5.1). A V-type H^+ -ATPase has also been discovered in the apical membranes of nephron segments that are positive for the putative ammonia transporter Rhp2 in the sinus zone (Nakada et al., 2010). Moreover, a H^+ , K^+ -ATPase was localized to apical membranes of proximal tubules, but basolateral membranes of intermediate tubules of dogfish (Swenson et al., 1994). Thus, “powerful protonation machinery” (Swenson et al., 1994) exists in the shark kidney even if it does not play a dominant role in acid–base regulation and very similar transporters have been retained in the mammalian kidney that does play a more significant role in systemic acid–base balance.

Besides direct H^+ secretion and HCO_3^- reabsorption, two buffer systems, phosphate, and NH_4^+ , are actually more important to renal acid–base balance than simple H^+ secretion (Lote, 1994). Secreted or filtered HPO_4^{2-} combines with a secreted H^+ to form H_2PO_4^- in the filtrate and the HCO_3^- generated in the tubule cell is reabsorbed back to the blood. H_2PO_4^- is excreted as $\text{NaH}_2\text{PO}_4^-$ and makes up a large portion of measured titratable acidity (TA) in the urine. In marine elasmobranchs, renal acid excretion is dominated by TA (King and Goldstein, 1983), and most of the TA is composed of phosphate (Swenson and Maren, 1986). Phosphate is thought to be mostly secreted, not filtered, in marine elasmobranchs (Swenson and Maren, 1986).

Ammonia is generated in renal tubules and secreted in the urine while HCO_3^- is reabsorbed back to the blood in vertebrates (reviewed by Weiner and Verlander, 2014). Whether NH_3 plus an H^+ or NH_4^+ directly are secreted, an H^+ is carried away in the urine. Ammonia is synthesized from the catabolism of amino acids (e.g., glutamine, glutamate) in elasmobranch tubule cells and renal ammonia excretion increases during a metabolic acidosis (King and Goldstein, 1983), as has been shown in all other major vertebrate groups. In *Squalus acanthias*, it is interesting that injection of HCl only decreased blood pH for ~30 min but renal ammonia excretion

remained elevated for more than 2 days. However, this elevated ammonia excretion made only a minor contribution to renal acid excretion relative to TA (<1%) (King and Goldstein, 1983). What is curious about the fact that marine elasmobranchs secrete ammonia during acid stress is that they are typically N limited. So why would such a system evolve and be retained over millions of years? Is it possible that the ammonia produced in the elasmobranch kidney during acidosis is simply a byproduct, whereas the amino acids provide important fuel (i.e., ATP) for active H⁺ and phosphate secretion? Dogfish kidney slices were able to generate NH₄⁺ from glutamine, glutamate, aspartate, alanine, and glycine and these amino acids were present in much higher levels (5-to-130-fold) than in the plasma (King and Goldstein, 1983). Over evolutionary time in terrestrial animals coping with excess N, this renal ammoniagenic strategy may have been co-opted as the main component of the renal response to metabolic acidosis. Answers to these evolutionary questions will never be known, but renal ammonia excretion during acidosis in marine elasmobranchs supplies only a very minor portion of the net acid secreted.

3.2.3. RECTAL GLAND

The NKA-rich MRCs of the gland contain abundant carbonic anhydrase (Maren, 1967; Lacy, 1983) and express NHE2 and NHE3 (Choe et al., 2005; Claiborne et al., 2008), Rh proteins (Nawata et al., 2015a), and soluble adenylyate cyclase (Roa et al., 2012; Tresguerres et al., 2014), so a role in acid-base balance might be expected. However, direct measurements of the acid-base composition of rectal gland secretion from *in vitro* (Shuttleworth et al., 2006), *in situ* (Swenson and Maren, 1984, 1987), and *in vivo* preparations (Shuttleworth et al., 2006; Wood et al., 2007c) of *Squalus acanthias* subjected to a variety of acid-base disturbances have shown clearly that this is not the case. The composition of the secretion only weakly reflects changes in plasma acid-base status such that its contribution to whole body acid-base balance is negligible. Nevertheless, rectal gland function is exquisitely sensitive to systemic acid-base status, as it is stimulated by alkalosis and inhibited by acidosis, effects which appear to be primarily mediated through extracellular pH, rather than PCO₂, [HCO₃⁻], or intracellular pHi (Swenson and Maren, 1984; Shuttleworth et al., 2006; Wood et al., 2007c). This sensitivity appears adaptive as low extracellular pH would inhibit gland function during post-exercise acidosis (Richards et al., 2003), thereby conserving extracellular fluid at a time of blood volume contraction, and high extracellular pH would stimulate gland NaCl and fluid output during the post-feeding “alkaline tide” (Wood et al., 2005, 2007a,b, 2009), a time of volume and salt loading (Wood et al., 2008).

3.2.4. GUT

The major acid–base events of the gastrointestinal tract are associated with feeding and include the secretion of HCl into the stomach compartments and the secretion of base, probably as HCO_3^- , into the intestine. In experimental studies on feeding in *Squalus acanthias*, vomiting of meals with accompanying loss of acidic equivalents to the environment has been reported (Kajimura et al., 2006; Wood et al., 2007a) but it is unclear whether this occurs in nature or is an artifact of experimental stress. Similarly, there is no information on whether base is normally excreted to the external water from the lower tract. However, Taylor and Grosell (2006) reported that HCO_3^- levels 10-fold greater than plasma levels were present in the intestine (“spiral valve”) of *Chiloscyllium plagiosum* when the animals were forced to drink by exposure to 140% seawater. Later reports indicated that similarly elevated intestinal HCO_3^- levels were present in the same species even when acclimated to 100% seawater (Anderson et al., 2007), as well as in three other elasmobranchs in 100% seawater (Anderson et al., 2010, 2012). Several *in vitro* gut sac studies have also shown that HCO_3^- secretion and water absorption occur in these same elasmobranchs acclimated to 100% seawater (Anderson et al., 2010; Liew et al., 2013), and surprisingly, neither rate increased in sac preparations of *Chiloscyllium plagiosum* acclimated to 140% seawater (Anderson et al., 2007). Thus, while intestinal HCO_3^- secretion definitely occurs in fasted animals, it is not clear if it is linked to osmoregulation. In teleosts, this system serves to precipitate Ca^{2+} and Mg^{2+} out of the intestinal fluid so as to aid water absorption in the posterior intestine (Wilson et al., 2002), and then this base is excreted in fluid leaving the colon. In this regard, it is noteworthy that on a whole animal basis, *Squalus acanthias* normally exhibits a small net excretion of basic equivalents to the environment under fasted conditions (Wood et al., 2007a, 2009). After feeding, intestinal HCO_3^- secretion appears to increase greatly, as outlined below.

In the first 12 h after voluntary or involuntary feeding, *Squalus acanthias* exhibited a marked “alkaline tide” (metabolic alkalosis; Brunton, 1933) in the bloodstream (Wood et al., 2005, 2007b, 2009) accompanied by a large net excretion of basic equivalents across the gills into the external water, the latter lasting more than 48 h (Wood et al., 2007a; Tresguerres et al., 2007). If this efflux had not occurred, blood pH would have risen by >0.8 pH units, which would undoubtedly have been fatal; indeed, the high capacity of the branchial base excreting mechanism (see Section 3.2.1) is probably designed for this purpose. Undoubtedly, the entire phenomenon is the consequence of accelerated HCl secretion in the stomach compartments, because all of these events (stomach acid secretion, blood alkalosis, and net base excretion to the

environment) were greatly attenuated by pre-treatment of the animal with omeprazole, a specific inhibitor of gastric H^+ , K^+ -ATPase (Wood et al., 2009). Future studies should assess whether the base excreted to the environment is taken up later (i.e., >48 h) to neutralize the acidic chyme as it enters the intestine, because digestion is a very slow process in elasmobranchs in general (see Chapter 6), taking greater than 5 days in this species (Wood et al., 2007b).

The gastric H^+ , K^+ -ATPase (Smolka et al., 1994; Choe et al., 2004) of the elasmobranch stomach facilitates secretion of H^+ , and Cl^- follows in equimolar amounts. The system is capable of achieving gastric fluid pH values well below 2.0 (Babkin, 1935; Hogben, 1959, 1967; Rehm, 1962, Kidder, 1976, 1991). The cellular basis (oxynticopeptic cells for combined acid and pepsinogen secretion vs. separate parietal and chief cells) seems to vary amongst species, and there appear to be different temporal secretory patterns, with either negligible HCl secretion during fasting (circum-neutral stomach pH) or continuous baseline secretion during fasting (low stomach pH) – see Chapter 6 for a discussion of these issues. Once food is ingested, HCl excretion is greatly augmented by adherents of both strategies. Chyme pH may be initially quite high because of buffering by the food, but it progressively drops to the pH 2.0–3.0 range as digestion proceeds (Wood et al., 2007b, 2009). Early *in vitro* studies on gastric HCl secretion in several elasmobranch species indicated that its electrophysiological characteristics were rather different from those in higher vertebrates (Hogben, 1959, 1967; Rehm, 1962; Kidder, 1976, 1991), but as yet there appears to have been no follow-up work on the finer details of the HCl secretory mechanism(s) with modern molecular physiology techniques in any elasmobranch.

In *Squalus acanthias*, once the acidic chyme (pH <4.0) moves into the intestine (“spiral valve”), its pH is precisely regulated at ~6.5, presumably by the addition of HCO_3^- (Wood et al., 2007b). Some of this HCO_3^- is undoubtedly secreted by the intestine itself, as greatly augmented HCO_3^- secretion rates were seen in intestinal sac preparations from fed versus fasted animals (Liew et al., 2013). Interestingly, this HCO_3^- secretion was eliminated by phloretin, a blocker of many transporters, including Cl^-/HCO_3^- exchangers. Additional sources may include the bile, though its HCO_3^- concentrations are quite low (Boyer et al., 1976; Wood et al., 2007b), and the large discrete pancreas (Holmgren and Nilsson, 1999), which is known to be a powerful organ of base secretion in higher vertebrates. However, the pancreas has not been investigated in this regard in elasmobranchs. This base secretion brings the HCO_3^- concentration approximately equal to that in the blood plasma, but as chyme pH is about 1.2 pH units below blood pH, chyme PCO_2 is very high (>20 Torr; Wood et al., 2007b). This may have important consequences in releasing O_2 by the Bohr effect from intestinal blood flow,

thereby enhancing O₂ supply to the working enterocytes. Gut acid–base physiology will be a fertile area for future research in elasmobranchs.

3.3. Freshwater and Intermediate Salinities

At present, information is sparse, restricted to studies on just one or a two species of each category (see [Section 2.3](#) for category definitions).

3.3.1. DILUTION TOLERATORS

In *Heterodontus portjacksoni* ([Cooper and Morris, 1998, 2004](#)) and *Squalus acanthias* ([Guffey and Goss, 2014](#)), transfer to dilute water was accompanied by transient increases in arterial blood P_aCO₂ and longer lasting increases in plasma [HCO₃⁻] and pH_a. The PaCO₂ increases were probably due to initial dilution of the hematocrit resulting in less efficient CO₂ excretion, whereas the [HCO₃⁻] and pH_a increases were symptomatic of the metabolic alkalosis encountered by almost all marine water-breathers when moving into lower salinities and usually attributed to Strong Ion Difference (SID) effects (differential permeability to cations versus anions; [Stewart, 1981](#)). In both studies, plasma [Cl⁻] was better regulated than plasma [Na⁺], perhaps because of compensation by gill Cl⁻/HCO₃⁻ exchange mechanisms. However there is no information on the gill acid–base exchange processes, or gut, kidney, and rectal gland acid–base responses to dilution in these animals, apart from a recent report of mRNA expression for the unusual NHE3k/i being upregulated in the intestine and down regulated in the kidney after acclimation of *Triakis scyllium* to decreasing salinity ([Li et al., 2013](#)).

3.3.2. FACULTATIVE FRESHWATER ELASMOBRANCHS

Freshwater acclimated stingrays (*Dasyatis sabina*) exhibited a metabolic alkalosis (higher plasma [HCO₃⁻] and pH_a, unchanged PaCO₂) relative to seawater-acclimated specimens ([Choe and Evans, 2003](#)), similar to the response in the dilution tolerators. When both groups were exposed to experimental hypercapnia, the seawater rays excreted acidic equivalents twice as fast as the freshwater animals, achieving a much more rapid and complete compensation of blood pH_a by the accumulation of plasma [HCO₃⁻]. This difference was attributed to the much higher Na⁺ levels in seawater, allowing more rapid acid excretion through the Na⁺/H⁺ exchange mechanisms at the gill. While this may well be true, it is interesting that virtually all elements of the potential acid and base extrusion mechanisms discussed in [Section 3.2.1](#) were more prominent in freshwater-acclimated animals. These included apparent proliferation of the NKA and VHA cells onto the secondary lamellae ([Fig. 5.2](#)) ([Piermarini and Evans, 2000, 2001](#);

Piermarini et al., 2002; Choe et al., 2005); greater NKA activity (Piermarini and Evans, 2000); increased mRNA expression of NHE3 (Choe et al., 2005) and H^+ , K^+ -ATPase (Choe et al., 2004) in NKA cells; greater protein abundance and more intense apical labeling of pendrin (Piermarini et al., 2002); and more intense cytoplasmic labeling of V-type H^+ -ATPase in VHA cells (Piermarini and Evans, 2001). Very similar differences were seen in freshwater- versus seawater-acclimated bullsharks, *Carcharhinus leucas* (Reilly et al., 2011). Presumably these acclimatory changes occur to facilitate active Na^+ , Cl^- , and K^+ uptake from the dilute external media. The slower acid-base compensation in freshwater could result either because the availability of external counterions remains limiting despite the increased transport machinery, or because the putative Na^+ versus acid and Cl^- versus base exchange processes are both running fast in parallel so as to facilitate necessary ion uptake, and therefore canceling each other out in terms of acid-base compensation. Simultaneous measurements of unidirectional ion fluxes and acidic equivalent fluxes would be needed to address this interesting issue.

Another interesting issue is the apparent large role played by NH_4^+ excretion in the freshwater rays, completely opposite to the situation in seawater rays. In the former, net acid excretion during the partial compensation of respiratory acidosis was entirely attributable to increased ammonia excretion, with no change in tritatable acid efflux (Choe and Evans, 2003).

This same study appears to be the only one to examine the role of the kidney in acid-base compensation in freshwater elasmobranchs. Although marine elasmobranchs excrete relatively little acid through their kidneys, this may be explained by the fact that they have a lower UFR relative to elasmobranchs in more dilute environments (Cooper and Morris, 2004). Thus, Choe and Evans (2003) asked whether renal acid excretion would contribute more to correcting a systemic acidosis in *Dasyatis sabina* acclimated to freshwater, where UFR is known to be exceptionally high, 2.5–10-fold greater than in freshwater teleosts (Janech and Piermarini, 2002). They found that renal TA- HCO_3^- , ammonia and net acid excretion were unchanged after freshwater acclimated fish were exposed to hypercapnia. However, they may have found different results if they had instead induced a metabolic acidosis, which is known to induce a small but significant increase in net acid and ammonia excretion in seawater-acclimated *Squalus acanthias* (King and Goldstein, 1983; see Section 3.2.2). In rainbow trout, metabolic acidosis is far more effective than respiratory acidosis in stimulating renal ammonia synthesis and excretion, and net acid excretion (Wood et al., 1999). Nonetheless, renal TA- HCO_3^- excretion might be expected to contribute to renal net acid excretion in

Dasyatis sabina under respiratory acidosis, as it does in trout (Wood et al., 1999), but this was not the case. Do euryhaline elasmobranch kidneys synthesize ammonia from amino acids? Is urine pH altered by acid or base loads and does this vary with acclimation to different external salinities? Does the gut or rectal gland play any role in acid–base regulation? There is much to find out in this interesting group.

3.3.3. OBLIGATE FRESHWATER ELASMOBRANCHS

The gills of freshwater potamotrygonid stingrays clearly have abundant NKA cells (Duncan et al., 2010, 2011), but VHA cells have not yet been identified. Nevertheless, radiotracer flux experiments with $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ have shown a vigorous branchial turnover of both ions (Carrier and Evans, 1973; Wood et al., 2002, 2003). Inhibition of unidirectional Na^+ uptake by amiloride analogues and of unidirectional Cl^- uptake by classical anion exchange inhibitors (thiocyanate and diphenylamine-2-carboxylic acid) suggests that Na^+ versus acid and Cl^- versus base exchange processes are both present (Wood et al., 2002). Interestingly, amiloride also transiently inhibited ammonia excretion, which indicates direct or indirect coupling to the acid excretion mechanism. These animals routinely encounter very low pH in the dissolved organic carbon (DOC)-rich blackwaters of the Rio Negro. In DOC-free reference water, unidirectional Na^+ and Cl^- influx rates were strongly inhibited and efflux rates accelerated by exposure to $\text{pH} = 4.0$. However, the DOC component of blackwater completely prevented the effects on efflux rates and partially prevented the decreases in influx rates (Wood et al., 2003). It would be interesting to know if DOC has similar protective effects on acid–base compensatory mechanisms. This, plus the presently unknown roles of the gut, kidney, and degenerate rectal gland (Thorson et al., 1978) in these processes are all open areas for future investigation.

4. NITROGENOUS WASTES

4.1. Overview

The end product of amino acid catabolism is ammonia in all animals. Ammonia exists in two forms in aqueous solutions; the dissolved gas, NH_3 and the ion, NH_4^+ . With a pK of 9.0–9.5, NH_4^+ dominates under physiological conditions. In this chapter, ammonia refers to both forms of ammonia and the chemical symbols are used to specify the specific form. In most fish, nitrogenous wastes are eliminated primarily across the gills as ammonia (reviewed by Wood, 1993; Wilkie, 1997; Ip et al., 2001; Ip and

Chew, 2010). Marine elasmobranchs are an interesting exception to this general rule because little ammonia is released to the environment and most of the nitrogen lost is in the form of urea (Goldstein and Forster, 1971a; Wood et al., 1995, 2007a; Steele et al., 2005), although there are species variations in the ratio of ammonia:urea excretion (Table 5.3).

Urea is synthesized in marine elasmobranchs, as well as holocephalons and coelacanth, and retained at relatively high concentrations (300–500 mmol L⁻¹) to counter balance the osmotic concentrations of the external seawater environment, the ureosmotic strategy of osmoconformation. Other organic solutes, especially methylamines (e.g., trimethylamine oxide (TMAO)) and/or β amino acids are also present in significant quantities and constitute important counteracting solutes that stabilize protein function (Yancey and Somero, 1980; Yancey et al., 1982; Somero, 1986; Ballantyne et al., 1987; Treberg et al., 2006) and phospholipid membranes (Barton et al., 1999) at elevated urea concentrations and may also play a role in

Table 5.3
Rate of total ammonia and urea excretion in elasmobranchs

| Species | Environment osmolality | Ammonia excretion (μmol N · kg ⁻¹ · h ⁻¹) | Urea excretion (μmol N · kg ⁻¹ · h ⁻¹) | Ratio ammonia: urea rates | Study |
|-------------------------------|------------------------|--|---|---------------------------|-------------------------------|
| Marine | | | | | |
| <i>Taeniura lymma</i> | 30 ppt | 120 | 740 | 0.16 | Ip et al. (2005) |
| <i>Hemiscyllium plagiosum</i> | 33 ppt | 110 | 1336 | 0.08 | Chan and Wong (1977b) |
| <i>Leucoraja erinacea</i> | 33 ppt | 140 | 400 | 0.35 | Steele et al. (2005) |
| | 100% SW | 111 | 480 | 0.23 | Goldstein and Forster (1971a) |
| <i>Squalus acanthias</i> | 30 ppt | 21 | 570 | 0.04 | Nawata et al. (2015b) |
| | 30 ppt | 28 | 549 | 0.05 | Wood et al. (1995) |
| | Fasted | 60 | 750 | 0.08 | Wood et al. (2007) |
| | Fed (10 h) | 112 | 1020 | 0.11 | Wood et al. (2007) |
| <i>Raja rhina</i> | 30 ppt | 145 | 1620 | 0.09 | Walsh et al. (2001) |
| Euryhaline | | | | | |
| <i>Himantura signifer</i> | 0.7 ppt | 390 | 144 | 2.71 | Chew et al., 2006 |
| | 15 ppt | 200 | 244 | 0.82 | |
| | 0.7 ppt | 230 | 200 | 1.15 | Tam et al. (2003) |
| | 20 ppt | 60 | 276 | 0.22 | |
| Freshwater | | | | | |
| <i>Potamotrygon</i> spp. | FW | 980 | ~100 | 9.8 | Goldstein and Forster (1971b) |

Abbreviations: SW, seawater; FW, freshwater

thermoprotection (Kolhatkar et al., 2014) and buoyancy (Withers et al., 1994). In general, the intracellular ratio of urea: TMAO is $\sim 2:1$ in many marine elasmobranchs (Yancey et al., 1982; Yancey and Somero, 1980), but in some species TMAO levels are not as high and other counteracting organic osmolytes contribute (e.g., Steele et al., 2005; Treberg et al., 2006) and this also depends on habitat depth (Laxson et al., 2011). At depth, high hydrostatic pressure destabilizes proteins and TMAO counteracts this effect (see Chapter 4). Therefore, nitrogenous compounds are essential for osmotic balance and the elasmobranch excretory system has evolved to prevent excessive loss of nitrogen. Ammonia is essential for the elasmobranch ureosmotic strategy of osmoregulation. In contrast, in teleost fishes, accumulation of ammonia is potentially toxic and it is continuously eliminated because most adult teleosts do not synthesize urea at high rates like elasmobranchs.

Many excellent reviews have been published on urea-based osmoregulation in marine elasmobranchs and readers are referred to Perlman and Goldstein (1988), Goldstein and Perlman (1995), Ballantyne (1997), Hazon et al. (2003), Hammerschlag (2006), McDonald et al. (2012) and Trischitta et al. (2012). In true freshwater elasmobranchs (*Potamotrygon* sp.), tissue urea levels and osmolality are low, similar to freshwater teleosts (reviewed by Ballantyne and Robinson, 2010). In contrast, euryhaline elasmobranchs in freshwater have significant levels of urea and other organic osmolytes, albeit at lower concentrations than when they are acclimated to seawater (reviewed by Ballantyne and Fraser, 2013). The euryhaline and freshwater species will be discussed at the end of this Section (see Section 4.3).

4.1.1. SITES AND MECHANISMS OF NITROGEN — METABOLITE PRODUCTION

The liver is the key site of amino acid catabolism and the formation of ammonia. Typically glutamate dehydrogenase (GDH) catalyzes the synthesis of glutamate from α -ketoglutarate and ammonia in a reversible reaction. However, GDH appears to be less important in supplying glutamate for the production of glutamine in marine elasmobranch mitochondria (Speers-Roesch et al., 2006) and other amino acids and transaminase enzymes may be important in producing glutamate (Ballantyne and Fraser, 2013). Glutamate and ammonia are converted to glutamine, catalyzed by glutamine synthetase, GS, the nitrogen-donating substrate for the first step in the ornithine-urea cycle (OUC) (Fig. 5.3; see also Chapter 4). Both GS affinity for ammonia (Shankar and Anderson, 1985) and absolute GS activities are exceptionally high in *Squalus acanthias* liver, kidney, and brain relative to other tissues (Chamberlin and Ballantyne, 1992), likely an effective strategy to scavenge ammonia for urea synthesis and also prevent neural toxicity from elevated ammonia.

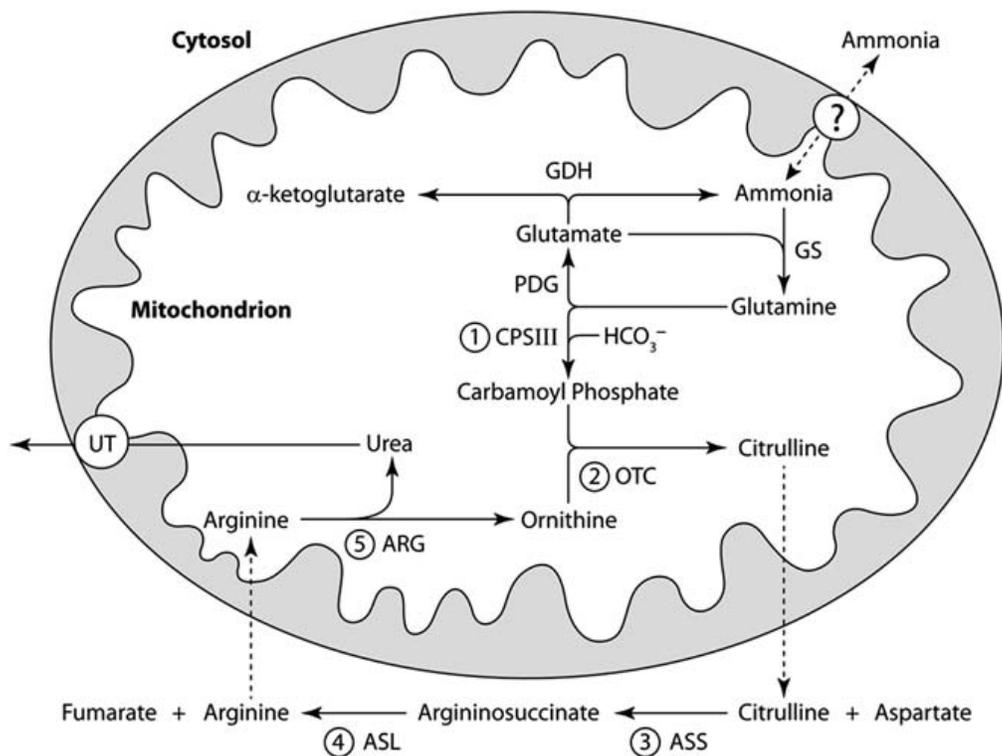


Figure 5.3. The ornithine-urea cycle (OUC) in a marine elasmobranch mitochondrion. Ammonia may enter or exit via an unknown carrier or be generated within the mitochondrion from the transamination of glutamate, catalyzed by glutamate dehydrogenase (GDH). Ammonia is also used along with glutamate to form glutamine, the N-donating substrate of the elasmobranch OUC, catalyzed by glutamine synthetase (GS). Glutamine and HCO_3^- form carbamoyl phosphate (step 1), catalyzed by carbamoyl phosphate synthetase III (CPS III) only found in fish. Carbamoyl phosphate and ornithine combine to form citrulline (step 2), catalyzed by ornithine transcarbamoylase (OTC). Citrulline leaves the mitochondrion to enter the cytosol where it combines with aspartate to form argininosuccinate (step 3), catalyzed by argininosuccinate synthetase (ASS). Argininosuccinate is split to form arginine and fumarate (step 4), catalyzed by argininosuccinate lyase (ASL). Arginine re-enters the mitochondrion where it is degraded to urea and ornithine (step 5), catalyzed by arginase (ARG). Ornithine re-enters the cycle, whereas urea is transported to the cytosol via a H^+ -linked phloretin sensitive urea transporter (UT). Also note that glutamine can also be recycled to glutamate, catalyzed by phosphate-dependent glutaminase (PDG). See text for further details.

Urea synthesis via the hepatic OUC was first demonstrated in marine elasmobranchs by [Schooler et al., \(1966; see also Chapter 7\)](#). However, more recently, low levels of OUC enzyme activities have also been detected in skeletal muscle tissue ([Steele et al., 2005; Kajimura et al., 2006](#)), stomach ([Tam et al., 2003](#)), intestine ([Kajimura et al., 2006](#)), and kidney of the closely related holocephalan elephant fish *Callorhynchus milii* ([Takagi et al., 2012](#)).

There have been numerous excellent reviews on the unique characteristics of the OUC in elasmobranchs (Anderson, 1991, 1995, 2001; Campbell and Anderson, 1991; Ballantyne, 1997; Ballantyne and Fraser, 2013). Mommsen and Walsh (1989) and Walsh and Mommsen (2001) provide an evolutionary perspective. Briefly, glutamine and HCO_3^- are combined to form carbamoyl phosphate, catalyzed by carbamoyl phosphate synthetase III (step 1, CPS III; Fig. 5.3). Note in other ureotelic vertebrates (e.g., mammals), CPS I requires ammonia as the N-donating substrate for carbamoyl phosphate synthesis (Anderson, 2001). Step 2 is catalyzed by ornithine transcarbamoylase (OTC) forming citrulline. Upon exiting the mitochondrion, citrulline along with aspartate, are converted to argininosuccinate, catalyzed by argininosuccinate synthetase (step 3, ASS). Argininosuccinate is then split into arginine and fumarate, catalyzed by argininosuccinate lyase (step 4, ASL). Arginine diffuses into the mitochondrion where the final step is completed and urea and ornithine are formed, catalyzed by arginase (step 5, ARG). Urea diffuses down the concentration gradient to the cytosol likely through a urea transporter (UT). In the little skate *Leucoraja erinacea*, urea uptake by mitochondria was phloretin-sensitive, saturable ($K_m = 0.34 \text{ mmol L}^{-1}$), and H^+ -linked, similar to bacterial urea transporters (Rodela et al., 2008). Note, phloretin is a well-established inhibitor of facilitated urea transport (Hays et al., 1977). To our knowledge, there is no information in the literature whether ammonia is transported across fish mitochondrial membranes as NH_4^+ or NH_3 and what type of transport protein is involved. Given that mitochondrial pH is higher than cytosolic pH, and ammonia is generated within the mitochondria, it is probable that excess ammonia leaves the mitochondria as NH_3 . Conversely, ammonia entering the mitochondria may be in the form of NH_4^+ due to the pH differences, but this is unknown.

Urea synthesis is relatively costly, requiring 5 ATPs per mole of urea (Kirschner, 1993). With respect to overall metabolic rate, Kirschner (1993) estimated that marine elasmobranchs use $\sim 10\%$ of the oxygen consumed for urea synthesis. Thus, it is not surprising that marine elasmobranchs conserve urea by reabsorbing filtered urea in the kidneys (Smith, 1931b) and have low gill permeability to urea (Pärt et al., 1998; Fines et al., 2001; Hill et al., 2004; Wood et al., 2013; see Section 4.2.1). Urea must be synthesized at sufficient rates to match what is lost, mostly across the gills (Wood et al., 1995).

OUC enzyme activities are modified to match the demand for urea. Hepatic OUC enzyme activities are altered with salinity changes in the environment (see Section 4.3). As well, infusion of NH_4Cl significantly elevated OUC activities in *Himantura signifer*, a semi-euryhaline

elasmobranch living in dilute environments in Southeast Asia that retains OUC capacity (Ip et al., 2003). Taken together these findings suggest that hepatic urea production is regulated through alterations in OUC enzyme activities in response to the changing demand for urea.

There has been controversy in the literature about the site and synthesis of the counterbalancing organic osmolyte, TMAO (see also Chapter 4). TMAO concentrations in marine elasmobranchs vary depending on the species and tissue type, ranging between 40–300 mmol L⁻¹ (Goldstein et al., 1967; Goldstein and Palatt, 1974; Withers et al., 1994; Bedford et al., 1998; Treberg et al., 2006; Wood et al., 2010; Laxson et al., 2011). For excellent detailed discussions of the possible pathways of methylamine synthesis readers are referred to Seibel and Walsh (2002) and Ballantyne and Fraser (2013). Briefly, TMAO can be obtained in the diet and some elasmobranch species that rely on dietary TMAO lack the capacity to synthesize it (Goldstein et al., 1967; Treberg and Driedzic, 2006; Treberg et al., 2006; Wood et al., 2010). TMAO can be produced from trimethylalkylammonium molecules (e.g., choline), which are converted to trimethylamine (TMA) before oxidation to TMAO, which requires gut bacteria. Alternatively, TMA can be synthesized in the mitochondrion of some marine animals after choline is converted to betaine aldehyde and then glycine betaine (Seibel and Walsh, 2002). There is considerable uncertainty, however, about the extent and exact pathway of TMAO biosynthesis in marine elasmobranch species (Goldstein and Palatt, 1974). It is clear that species that retain elevated TMAO concentrations reabsorb TMAO in the kidney (Cohen et al., 1958; Goldstein and Forster, 1971a) and lose very little even after prolonged fasting (Cohen et al., 1958; Treberg and Driedzic, 2006; Kajimura et al., 2008; Wood et al., 2010).

Amino acids play an important role in intracellular osmotic balance in marine elasmobranchs (Forster and Goldstein, 1976; King et al., 1980; Goldstein, 1981). A genomics survey of *Squalus acanthias* found that amino acid metabolic enzymes dominate in osmoregulatory tissues (Lee et al., 2006). In the skeletal muscle of *Leucoraja erinacea*, taurine, betaine, and β -alanine are found in concentrations between 20–40 mmol L⁻¹ each, but in liver tissue only taurine (50 mmol L⁻¹) is found at much higher concentrations relative to other amino acids (Steele et al., 2005). Goldstein (1981) proposed that these amino acids may be valuable in osmoregulation because they are not used in protein synthesis. Creatine, a phosphogenic compound used in ATP production, is also present in significant levels (10–35 mmol L⁻¹) in skeletal muscle tissue of many marine elasmobranchs and appears to be regulated with environmental salinity and depth (Steele et al., 2005; Laxson et al., 2011).

4.2. Seawater

4.2.1. GILLS

The gills are the major site of nitrogen release in marine elasmobranchs (Payan et al., 1973; Wong and Chan, 1977; Wood et al., 1995). Urea excretion dominates over ammonia excretion in marine elasmobranchs (Table 5.3).

Gill urea permeability is much lower in marine elasmobranchs than in teleost fishes, which is especially interesting because of the very high plasma-to-water urea gradient in elasmobranchs (Boylan, 1967). The reason for this may relate to the composition of the lipid bilayer of branchial epithelium, the presence of an active urea transporter, and/or the relatively low surface area of the apical membrane. Fines et al. (2001) reported that the basolateral membrane of gill epithelia in *Squalus acanthias* had a higher phosphatidylcholine-to-phosphatidylethanolamine and cholesterol-to-phospholipid molar ratio than other fish membranes. Both features would stabilize the membrane and the elevated cholesterol would decrease the permeability of the intact gill to urea. Apical gill membranes in *Squalus acanthias* have an even lower urea permeability than basolateral membranes and coupled with a low surface area, these would represent an even greater barrier to urea loss (Hill et al., 2004). However, there is also evidence for an active Na^+ -coupled urea “back transporter” that would return part of the urea lost to the gill cell back to the plasma against the urea gradient (Pärt et al., 1998; Fines et al., 2001). Working with an isolated gill basolateral membrane preparation from *Squalus acanthias*, Fines et al. (2001) showed that urea transport was phloretin-sensitive and Na^+ -coupled ($K_m = 10 \text{ mmol L}^{-1}$). The low K_m value implies that urea levels in gill epithelial cells might be much lower than the blood. However this appears not to be the case, with recent measurements showing similar concentrations in the two compartments (Wood et al., 2013). Furthermore, using an isolated perfused head preparation in *Squalus acanthias*, Wood et al. (2013) demonstrated that urea efflux was inhibited by equimolar concentrations of the urea analogs, thiourea or acetamide, which provided evidence for a urea transporter also on the apical membrane. The facilitated urea transporter in *Squalus acanthias* (ShUT) similar to the mammalian UT-A2 (You et al., 1993; reviewed by Sands, 2003) is strongly expressed at the mRNA level in kidney and brain, but not in gills (Smith and Wright, 1999), whereas the Na^+ -coupled urea transporter described in gills by Fines et al. (2001) is probably a very different protein. It is interesting that three UT isoforms have been identified in the holocephalon ratfish, *Hydrolagus colliei*, but in gill only UT-3 mRNA was detected (Anderson et al., 2012). So far no one has isolated a urea transporting protein in elasmobranch gills. There may be more than one urea transporter in the gill

tissue of marine elasmobranchs with different characteristics to ultimately prevent urea loss, a good avenue for further research.

Branchial urea excretion is influenced by environmental salinity (see Section 4.3) and the internal physiological state of the fish. Infusion of NH_4Cl or NaHCO_3 in intact *Squalus acanthias* significantly elevated the rate of branchial urea excretion without altering the plasma urea levels (Wood et al., 1995; Nawata et al., 2015b). In the blue-spotted fantail ray, *Taeniura lymma*, NH_4Cl injection into the peritoneal cavity also increased total body urea excretion (as well as ammonia excretion) without altering urea levels in most tissues (Ip et al., 2005). It is most likely that the changes in urea elimination were associated with increased rates of urea synthesis at least in *Squalus acanthias*, as both NH_4^+ (via glutamine production) and HCO_3^- are substrates for the OUC (Wood et al., 1995). Elevated environmental ammonia (1 mmol L^{-1}) for 48 h increased urea excretion rates in *Squalus acanthias* by 90%, surpassing the amount of ammonia that had been absorbed from the water (Nawata et al., 2015a). Thus, dogfish sharks are unable to store urea and the OUC appears to be over stimulated when the animal is loaded with ammonia (Nawata et al., 2015a,b). Working on the skate *Leucoraja erinacea*, Cameron (1986) earlier found that ammonia built up in the blood slowly during a similar high environmental exposure and speculated that this occurred because the ammonia was being converted to urea by the OUC. With acute severe hypoxia, total urea excretion was also significantly elevated while there were no changes in plasma and tissue urea concentrations (Zimmer et al., 2014). The authors suggest that low oxygen leads to a decline generally in gill homeostatic mechanisms. It would be interesting to know if gill urea transporter expression is altered under these conditions; however, such studies must await the molecular characterization of the gill urea transport protein(s).

The mechanisms involved in branchial ammonia excretion in elasmobranchs are unclear. Ammonia excretion across the branchial epithelium of teleost fishes is facilitated by a family of ammonia transporters, the Rhesus (Rh) glycoproteins (Wright and Wood, 2009). There is considerable evidence in freshwater teleosts for the model that Rh proteins facilitate NH_3 diffusion down the NH_3 partial pressure gradient and several associated transporters (e.g., NHE, H^+ -ATPase, carbonic anhydrase) form a metabolon with Rh proteins to move H^+ ions to the apical side of the gill while taking up Na^+ (Wright and Wood, 2012; Ito et al., 2013), but less is known about marine fishes. There is evidence that basolateral Rhbg is expressed in gill tissue of the shark *Squalus acanthias* at the mRNA level (Nawata et al., 2015a). The isoform Rhp2 was not detected at the mRNA level in the gills of the shark *Triakis scyllium* (Nakada et al., 2010), but was found in *Squalus acanthias* (Nawata et al., 2015a). Before the discovery of

Rh proteins researchers were debating whether NH_4^+ efflux was coupled to Na^+ influx in the gills of marine elasmobranchs (Payan and Maetz, 1973; Evans et al., 1979, 1988; Evans, 1982; Claiborne and Evans, 1992; see Sections 2.2.1 and 3.2.1). This question needs to be revisited in light of the discovery of multiple isoforms of the Na^+/H^+ exchanger, NHE (Choe et al., 2002; Edwards et al., 2002; Choe et al., 2005; Claiborne et al., 2008; Reilly et al., 2011; Li et al., 2013), as well as H^+ -ATPase (Tresguerres et al., 2005, 2006; Reilly et al., 2011) and putative Rh proteins that may be expressed in the same branchial cells of *Squalus acanthias*. Moreover, the localization of H^+ -ATPase to the basolateral membrane especially during alkalosis in *Squalus acanthias* (Tresguerres et al., 2006) suggests that facilitated NH_3 transport via Rh proteins in marine elasmobranchs gills may in some cases be directed to reabsorption from the gill cell to the blood, as has been proposed in the shark kidney (Nakada et al., 2010). These possibilities will provide an exciting area for future research.

Ammonia efflux in marine elasmobranchs is highly responsive to changes in blood acid–base status and ammonia gradients, but not external salinity. Infusions of NH_4Cl and/or NH_4HCO_3 in sharks enhanced ammonia excretion rates (Payan and Maetz, 1973; Wood et al., 1995; Nawata et al., 2015b), likely due to the large elevation of the blood-to-water NH_3 partial pressure gradients (P_{NH_3}). It is interesting that hypercapnia (24h) caused a marked increase in whole animal ammonia excretion in *Squalus acanthias* (likely across the gills; Claiborne and Evans, 1992), but not in *Dasyatis sabina* in seawater (Choe and Evans, 2003). The seawater pH in both studies was sharply decreased, enhancing the diffusion gradient for NH_3 and thus, an increase in ammonia efflux would be expected. It would be interesting to take a closer examination of branchial ammonia transport mechanisms between these two species. A reversal of the normal blood-to-water ammonia gradient was imposed when *Squalus acanthias* were exposed to elevated environmental ammonia (1 mmol L^{-1}) and it took 36 h before net ammonia excretion was re-established (Nawata et al., 2015a). Branchial Rhbg (not Rhp2) and erythrocytic Rhag mRNA levels declined, possibly to reduce ammonia uptake, and the expression of other transporters in the teleost Rh metabolon (e.g., carbonic anhydrase, NHE2) were unaltered, suggesting that the mechanism(s) of gill ammonia transport may be very different in elasmobranchs, relative to teleosts.

4.2.2. KIDNEYS

The nephron of the marine elasmobranch kidney is highly complex and arranged in a countercurrent flow arrangement between peritubular capillaries and the filtrate (Dantzler, 1989). A single nephron is partly

encapsulated in a peritubular sheath (bundle zone) and partly free within a blood sinus (sinus zone) (Lacy and Reale, 1985; reviewed by Henderson et al., 1988). This intricate arrangement is thought to facilitate the reabsorption of up to 99.5% of the filtered urea (Kempton, 1953; Boylan, 1972; Lacy and Reale, 1995). Numerous studies have shown that marine elasmobranchs carefully regulate renal urea reabsorption with profound (up to 25-fold) increases in urea clearance when fish were chronically exposed to a 50% dilution of their environment (Goldstein et al., 1968; Goldstein and Forster, 1971a; Payan et al., 1973; Wong and Chan, 1977; Janech et al., 2006b; see Section 4.3). However, even though some of these discoveries were made almost 50 years ago, we still do not have a broad understanding of the mechanisms involved.

Boylan's passive model of urea transport was proposed prior to the isolation of facilitated bi-directional urea transporters in the late 1990s. Boylan (1972) proposed that urea could be reabsorbed passively if a concentration gradient existed in a specific nephron segment. He further postulated that the terminal segment has a low water but high urea permeability and comes into close apposition with the first loop, which has a low urea permeability, allowing urea to move down its concentration gradient into the interstitial fluid. It is somewhat remarkable that no one has directly tested Boylan's hypothesis. The key to deciphering the relationships between the various nephron structures is to use micropuncture or isolated perfused tubules, in conjunction with immunohistochemistry to determine flux rates and localize the transporters within the nephron and at the cellular level. Except for some early micropuncture work by Schmidt-Nielsen et al. (1966) and Stolte et al. (1977), as well as perfusion studies on ion flux (Friedman and Hebert, 1990; Hebert and Friedman, 1990), there has been little progress in characterizing the transport properties of each nephron segment. However, there have been advances in our understanding of urea transport mechanisms.

Evidence for carrier-mediated urea transport in elasmobranch kidneys was first reported by Schmidt-Nielsen and Rabinowitz (1964). In *Squalus acanthias* the urea transport inhibitor, phloretin, partially blocked urea reabsorption (Hays et al., 1977) and there was a tight correlation between urea: Na⁺ reabsorption (1:1.6; Schmidt-Nielsen et al., 1972). In *Squalus acanthias*, a UT was first isolated (ShUT) from the kidney that was inhibited by phloretin and had 66% identity to the rat UT-A2 (Smith and Wright, 1999). Renal UTs have now been isolated in several other elasmobranchs (Janech et al., 2008; Anderson et al., 2010). In Atlantic stingray *Dasyatis sabina*, urea transport via strUT was sensitive to phloretin and urea analogs (Janech et al., 2003). Immunohistochemical localization of UT in the kidney of *Triakis scyllia* demonstrated that only the collecting

tubules of the bundle zone expressed this protein (Hyodo et al., 2004) (Fig. 5.1), supporting the idea of high urea permeability in this final segment proposed by Boylan (1972).

There is molecular and physiological evidence that more than one type of urea transporter may be involved in urea reabsorption in the elasmobranch kidney. Urea uptake by isolated renal brush border membrane vesicles of *Leucoraja erinacea* revealed that a phloretin-sensitive, nonsaturable uniporter was present in the dorsal section of the kidney, whereas a phloretin-sensitive Na⁺-dependent urea transporter was operating in the ventral section (Morgan et al., 2003b), supporting the earlier studies by Schmidt-Nielsen et al. (1972). Multiple UT transcripts have been isolated and characterized in *Leucoraja erinacea* (Morgan et al., 2003a) and *Dasyatis sabina* (Janech et al., 2006a), as well as in the holocephalan ureosmotic elephant fish (*Callorhinchus milii*; Kakumura et al., 2009) and there is evidence that they may have different functional properties. For example, environmental dilution decreased the expression of some but not all of the UT transcripts in *Dasyatis sabina* (Janech et al., 2006a). Thus, it seems likely that urea transporters with different functional properties, possibly localized in different renal tubule segments are involved in urea reabsorption in the kidney of marine elasmobranchs. Whether urea reabsorption is a completely passive process as Boylan (1972) suggested is still debatable, especially since there is evidence of Na⁺-linked urea transport.

The expression of UTs in the kidney are regulated with environmental dilution and ammonia loading. At the mRNA and protein level, low salinity generally decreases UT expression in the kidney of marine elasmobranchs (see Section 4.3). When *Squalus acanthias* were placed in a high ammonia environment, renal UT mRNA expression was enhanced by ~6-fold by 24 h associated with a rise in whole body urea excretion (Nawata et al., 2015a). NH₄Cl infusion caused similar responses (Nawata et al., 2015b). It is clear that renal UTs are responsive, possibly to plasma urea levels, but further studies are required to understand the role of these proteins in urea balance within the complex nephron arrangement.

There was some early confusion in the literature about whether elasmobranch erythrocytes possess a urea transporter. The first investigation by Murdaugh et al. (1964) led to the conclusion that urea transport was carrier-mediated; however, their methods were rather rudimentary. Using a more sophisticated approach with ¹⁴C-urea and inhibitors, these earlier results were later refuted (Rabinowitz and Gunther, 1973; Kaplan et al., 1974; Walsh et al., 1994; Carlson and Goldstein, 1997). As well, there was no expression of ShUT mRNA in erythrocytes of *Squalus acanthias* (Smith and Wright, 1999). Taken together it seems likely that urea transport across erythrocytes is by simple diffusion.

Compared to urea, less attention has been paid to ammonia transport in the kidney of elasmobranchs. Plasma ammonia levels were $\sim 80 \mu\text{mol L}^{-1}$ and urine levels were more than 3-fold higher ($290 \mu\text{mol L}^{-1}$) in cannulated, resting *Squalus acanthias*, indicating that ammonia is not always reabsorbed (Wood et al., 1995). Normally, the rate of ammonia excretion via the kidney is very low relative to renal urea excretion (ammonia $0.06 \mu\text{mol N kg}^{-1} \cdot \text{h}^{-1}$ versus urea $26 \mu\text{mol N kg}^{-1} \cdot \text{h}^{-1}$ in *Squalus acanthias*; Wood et al., 1995). However, renal ammonia excretion increases during a metabolic acid load (see Section 3.2.2) and ammonia is probably synthesized in the renal tubule cells from the catabolism of glutamine and glutamate under these conditions (King and Goldstein, 1983). The mechanisms of ammonia metabolism and transport in the kidney in response to acid-base imbalance is not well understood in elasmobranchs but is an important area for investigation from an evolutionary (appears to be a conserved response in vertebrates) and environmental point of view (ocean acidification).

It is likely that Rh proteins play a fundamental role in renal ammonia transport. Nakada et al. (2010) were the first to show that the Rh isoform Rhp2 mRNA was localized to the renal tubule cells in the second and fourth loop of the sinus zone of *Triakis scyllium* (Fig. 5.1). Rhp2 was detected in the basolateral membrane and the authors proposed that NH_3 reabsorption via Rhp2 was coupled to apical NH_4^+ transport via Na^+ , K^+ , 2Cl^- cotransporter, with the H^+ returned to the urine via H^+ -ATPase (Nakada et al., 2010). Interestingly Rhp2 mRNA levels in the kidney were elevated at high relative to low salinity (Nakada et al., 2010), but there were no changes in response to increased plasma and environmental ammonia levels in *Squalus acanthias* (Nawata et al., 2015a,b). The basolateral isoform, Rhbg, has also been detected at the mRNA level in the kidney, intestine, and rectal gland in *Leucoraja erinacea* (Anderson et al., 2010) and in the kidney of *Squalus acanthias*, but it also shows no significant response to elevated plasma ammonia (Nawata et al., 2015a,b). The apical isoform Rhcg found in teleost kidney (Nakada et al., 2007; Cooper et al., 2013; Wright et al., 2014) has not been detected in elasmobranchs, but homologous sequences appear in the *Squalus acanthias* transcriptome (A. Clifford and G. Goss, personal communication). Much work is required to isolate, characterize, and localize the full suite of Rh isoforms in the kidney and understand how they work with other transporters to regulate ammonia reabsorption and excretion in elasmobranchs. Because Rh proteins appear to co-localize with other acid-base/ion transporters in teleost and mammalian kidney tubules (Cooper et al., 2013; Wright et al., 2014; Weiner and Verlander, 2014), it will be interesting to learn if Rh-positive renal cells in elasmobranchs also express NHE and/or H^+ -ATPase isoforms (Bevan et al., 1989; Nakada et al., 2010; Li et al., 2013).

TMAO excretion is a relatively minor nitrogenous waste, constituting only 1–2% of the total nitrogen excreted (Treberg et al., 2006). TMAO is present in the plasma and other body fluids at much higher concentrations than in urine. From data reported by Cohen et al. (1958), the urine:plasma TMAO ratios in *Squalus acanthias* are 0.02–0.2, meaning that very little TMAO escapes in the urine. Such tight regulation of renal TMAO reabsorption is necessary if TMAO is solely obtained in the diet or synthesized at the cost of ATP. In dilute seawater, plasma and tissue TMAO levels decrease (Cooper and Morris, 1998; Steele et al., 2005), but exactly how TMAO reabsorption in the elasmobranch kidney is regulated has not been investigated to our knowledge. It is known that erythrocyte TMAO levels are controlled, in part, by a volume-activated osmolyte pathway (Wilson et al., 1999; Koomoa et al., 2001), but the molecular characterization is unknown.

4.2.3. RECTAL GLAND

Urea is reabsorbed in the rectal gland. Burger and Hess (1960) reported that the urea rectal gland fluid: plasma ratios in *Squalus acanthias* were ~0.05, indicating a minimal loss of urea by this route. The permeability of the rectal gland basolateral membrane is relatively low (Zeidel et al., 2005), suggesting that a secondary active carrier-mediated urea transporter may return urea to the blood against the urea gradient, as has been proposed in the gills (Fines et al., 2001). The renal UT does not appear to be present in rectal gland tissue (Smith and Wright, 1999; Janech et al., 2003; Hyodo et al., 2004; Janech et al., 2006a), maybe not surprising as it is thought to be a bi-directional uniporter. There are many gaps in our understanding of how the rectal gland reabsorbs urea and whether the urea transport mechanism (s) is linked to Na⁺ secretion. Furthermore, there is only very preliminary evidence that Rhbg and Rhp2 mRNA are expressed in rectal gland tissue, but there is no knowledge of cellular distribution and ammonia transport mechanisms (Nawata et al., 2015a). Investigations of urea and ammonia transport in elasmobranch rectal gland should be both fruitful avenues for research.

4.2.4. GUT

The majority of elasmobranchs are carnivores, with a smaller number of species consuming plankton (see Chapter 6). Thus, in most elasmobranchs the gastrointestinal tract morphology and secretions have evolved to digest large protein meals. As a consequence of protein and amino acid digestion, ammonia concentrations in the plasma increase after feeding (Wood et al., 2005, 2010; Kajimura et al., 2008), along with an increase in OUC enzyme activities (Kajimura et al., 2006). For a more detailed

discussion of the influence of feeding on elasmobranch homeostasis, readers are referred to Chapter 6. Here we provide a discussion on urea, ammonia, and TMAO handling in different gut sections.

The osmolality of plasma and fluids secreted along the gastrointestinal tract are similar, but there are marked differences between plasma urea and TMAO concentrations relative to those in the gastrointestinal fluids along the stomach, intestine, and colon. For example, the urea concentration in stomach fluid of the three fasted species, *R. eglanteria*, *Leucoraja erinacea*, and *Chiloscyllium plagiosum* (Anderson et al., 2010) and one fed shark, *Squalus acanthias* (Wood et al., 2007b) was 63–75% lower relative to the plasma. More astounding yet, the colon fluid was isosmotic with the blood but contained 0–5% of the urea found in the plasma (Anderson et al., 2010). In the skate *Leucoraja erinacea*, TMAO was also consistently lower (by $\geq 80\%$) than plasma across all gut sections (Anderson et al., 2010). Hence, major reabsorptive processes occur in the gut to conserve nitrogenous compounds (reviewed by Wood and Bucking, 2011).

Different approaches have been used recently to understand how the gastrointestinal tract osmoregulates between the fasted and fed state (see also Section 2.2.4). Whole shark studies have revealed that chyme osmolality is elevated by the secretion of urea, Na^+ , and Cl^- in the stomach when *Squalus acanthias* are eating hyposmotic marine teleosts (Wood et al., 2007b). In the intestine, more urea-rich fluid was secreted, possibly from the bile, pancreas, or intestinal wall itself before reabsorption of urea, Na^+ , Cl^- , and water in the late segments. However, it is difficult to address specific mechanisms of urea transport *in vivo*. The *in vitro* gut sac preparation (Liew et al., 2013) and Ussing chamber approach (Anderson et al., 2012, 2015; and Chapter 8) have provided a more detailed picture. While the gut sacs of fasted *Squalus acanthias* secrete urea across the intestinal wall, they reabsorb urea, Na^+ , Cl^- , and water at low rates in the stomach and colon (Liew et al., 2013). Upon feeding, the *in vitro* reabsorption of urea in the intestine was markedly enhanced and this flux was sensitive to phloretin. Coupled with the detection of UT mRNA in the intestine of *Leucoraja erinacea* (Anderson et al., 2010), there is growing evidence that intestinal urea reabsorption is carrier-mediated in marine elasmobranchs. *In vitro* intestinal urea flux was saturable ($K_m \sim 290 \mu\text{mol L}^{-1}$), partially inhibited by urea analogs, and Na^+ -dependent in *Squalus acanthias* (see Chapter 8). The authors concluded that it is likely that multiple transport pathways are available for urea reabsorption in the elasmobranch gut, based on their flux data and the fact that urea retention is of prime importance.

Ammonia is present in the gut of marine elasmobranchs, but few studies have measured flux or even concentrations. Lloyd and Goldstein (1969)

reported that in intestinal sacs of *Squalus acanthias* incubated *in vitro* there was bacterial degradation of urea forming ammonia, although the estimated rate was relatively low at 4 μ moles urea degraded per kg fish per hour. In a follow-up study, intact skates (*Leucoraja erinacea*) were administered antibiotics, which had no impact on plasma urea levels or urea excretion rates, but did eliminate ammonia synthesis in intestinal fluids (Goldstein and Dewitt-Harley, 1972). It appears then that ammonia is routinely generated by microbes in the gut, albeit at low levels. Chyme ammonia levels were 1–3 mmol L⁻¹ in *Squalus acanthias* (Wood et al., 2009). This is substantial and possibly a result of marine teleost fish consumption where intracellular muscle ammonia levels can be ~6–9 mmol L⁻¹ (Wright et al., 1988). When ~2 mmol L⁻¹ of ammonia was added to the mucosal side of a *Squalus acanthias* isolated colon epithelial preparation in an Ussing chamber, the flux to the serosal side was relatively low, which implied low tissue permeability (Anderson et al., 2012). Anderson et al. (2010) found Rhbg mRNA expression in the anterior, mid, and posterior intestine in the skate *Leucoraja erinacea*, with highest expression levels in the posterior segment. However, knowledge of the level of protein expression of Rhbg and other Rh isoforms, as well as cell localization is unknown. There may very well be regional differences in Rh protein expression along the gut that may determine ammonia permeability, which are fascinating avenues for future work.

4.3. Freshwater and Intermediate Salinities

4.3.1. DILUTION TOLERATORS

Many studies over the years have examined the impact of environmental dilution on urea metabolism and excretion in marine elasmobranchs, even though some of the species studied infrequently encounter low salinity. In general, dilute seawater (50–75%) lowers plasma and tissue urea, TMAO, and ion concentrations (Smith, 1931a; Goldstein and Forster, 1971a; Haywood, 1973; Payan et al., 1973; Chang and Wong, 1977a,b; Wong and Chan, 1977; Morgan et al., 2003a; Steele et al., 2005). Total body urea excretion rates in dilute seawater vary between species and studies. For example, in the little skate *Leucoraja erinacea*, exposure to 50% seawater (5 days) decreased (Goldstein and Forster, 1971a) and 75% seawater (25 ppt, 3–4 days) increased total body urea excretion rates (Steele et al., 2005). In the lip shark *Hemiscyllium plagiosum*, 46% seawater (15 ppt \geq 1 week) did not significantly alter total urea loss (Chan and Wong, 1977b; Wong and Chan, 1977). In *Squalus acanthias*, total urea efflux increased several fold over 48 h of exposure to 21‰ salinity (Guffey and Goss, 2014). Regardless of the species, renal excretion and clearance are markedly increased with

environmental dilution (3- to 24-fold; Goldstein et al., 1968; Goldstein and Forster, 1971a; Wong and Chan, 1977). Urea excretion and clearance by the rectal gland does not change very much with acclimation to a dilute seawater environment, unlike the large increases observed in the kidney (Wong and Chan, 1977). Low salinity decreased the level of UT mRNA of all transcripts in *Leucoraja erinacea* (Morgan et al., 2003a) and some UT transcripts in *Dasyatis sabina* (Janech et al., 2006a), whereas there were no significant changes at the mRNA level in *Triakis scyllium* kidney (Yamaguchi et al., 2009). At the protein level, apical UT in renal collecting tubules of *Triakis scyllium* was markedly decreased in response to dilute seawater exposure (Yamaguchi et al., 2009). Hepatic OUC activities increased in response to increased salinity (Tam et al., 2003) or decreased in response to dilute seawater (Steele et al., 2005). In skate (*Leucoraja erinacea*) and shark (*Hemiscyllium plagiosum*) at least 5 days of exposure to dilute seawater (down to ~15‰) did not alter total body ammonia excretion rates (Goldstein and Forster, 1971a; Chan and Wong, 1977b; Steele et al., 2005), but during the recovery phase in skates ammonia excretion rates were depressed initially, no doubt because ammonia was required to replenish the urea stores (Steele et al., 2005). Hence, even truly marine elasmobranchs respond to changes in environmental salinity and are able to regulate urea balance through changes in urea metabolism and transport.

TMAO plasma and tissue concentrations are also regulated to respond to environmental osmolality with decreasing levels after several days in a dilute environment (Goldstein et al., 1968; Goldstein and Forster, 1971a; Steele et al., 2005). Renal TMAO clearance and excretion increased 12- and 2- fold, respectively in *Leucoraja erinacea* in 50‰ seawater (Goldstein and Forster, 1971a). Other nitrogenous osmolytes such as β -alanine and creatine in skeletal muscle are also decreased with acclimation to reduced salinity in *Leucoraja erinacea* (Steele et al., 2005), presumably also through enhanced renal excretion, but this is unknown.

4.3.2. FACULTATIVE FRESHWATER ELASMOBRANCHS

A number of truly euryhaline elasmobranch species found along a salinity gradient maintain relatively high urea levels and the capacity to synthesize urea (e.g., De Vlaming and Sage, 1973; Tam et al., 2003; Pillans and Franklin, 2004). The stingray *Dasyatis sabina* has been a favorite model for understanding how euryhaline elasmobranchs regulate nitrogen balance when moving between marine, brackish, and freshwater environments (found in nature between 2 and 36.7 ppt; De Vlaming and Sage, 1973). In freshwater (1 ppt), they maintain substantial urea ($\sim 150 \text{ mmol L}^{-1}$) and methylamine levels ($\sim 75 \text{ mmol L}^{-1}$ mostly as

TMAO) in skeletal muscle (Treberg et al., 2006). When they were transferred from full strength seawater (1018 mOsmol L⁻¹) to a dilute environment (262 mOsmol L⁻¹, 26% seawater), plasma NaCl levels declined to a much greater extent than urea levels (De Vlaming and Sage, 1973), confirmed in later studies as well (Janech et al., 2006b). Although they held on to urea, increases in both glomerular filtration rate and urine flow rate resulted in an almost 20-fold increase in renal urea clearance and a lower fractional urea reabsorption when *Dasyatis sabina* were acclimated to 462 mOsmol L⁻¹ (Janech et al., 2006b). These changes were accompanied by lower transcript levels of one of the strUT renal isoforms, potentially resulting in lower expression at the protein level, but this was not determined (Janech et al., 2006a). To our knowledge, OUC enzyme activity has not been measured in this species to determine if these changes are accompanied by a down-regulation of urea synthesis. However, urea production by isolated hepatocytes has also been reported to increase close to three-fold when the euryhaline bull shark *Carcharhinus leucas* were acclimated to seawater (Anderson et al., 2005).

An interesting “intermediate” between the true stenohaline freshwater potamotrygonid stingrays, and the true euryhaline freshwater stingrays, may be *Himantura signifier*, a freshwater stingray of Southeast Asia. *Himantura signifier* tolerates only up to ~60% seawater (~20 ppt) yet in freshwater it retains a functional OUC and moderately high urea levels in the body fluids (40–80 mmol L⁻¹) (Tam et al., 2003; Ip et al., 2005). Urea concentrations double in the plasma, as well as increase in other tissues when these stingrays were transferred from 0.7 ppt to 15 or 20 ppt, indicating that *Himantura signifier* have the ability to regulate urea levels in the face of salinity challenge (Tam et al., 2003; Chew et al., 2006). Hepatic OUC activities in *Himantura signifier* were also significantly higher with acclimation to 20 ppt seawater (Tam et al., 2003) or exposure to elevated environmental ammonia (Ip et al., 2003). It is also interesting that a complete OUC was found in the stomach and CPS III activity was enhanced in this organ at higher salinities but not in response to ammonia. A fuller exploration of the functional significance of urea synthetic capacity in the stomach during feeding and fasting is necessary in this species, plus a comparison with other marine and euryhaline species would be valuable to determine if stomach urea synthesis is widespread.

4.3.3. OBLIGATE FRESHWATER ELASMOBRANCHS

Smith (1931a) reported urea levels in “freshwater” elasmobranchs; however, the species chosen were facultative freshwater species (i.e., truly

euryhaline) with ureagenic capacity. Not until more than 30 years later did [Thorson \(1967\)](#) discover the very low urea content in the obligate freshwater *Potamotrygon* spp. of the Amazon basin. These fish also lacked detectable levels of TMAO ([Thorson, 1967](#); [Treberg et al., 2006](#)). [Goldstein and Forster \(1971b\)](#) showed that *Potamotrygon* lacked sufficient CPS III for a functional urea cycle in the liver and were unable to retain urea following intravenous injection of ^{14}C -urea. This is consistent with a simpler kidney structure that lacks the tubular bundles found in marine species ([Lacy and Reale, 1995](#)). When gradually acclimated to 40% seawater (13‰), tissue and plasma urea levels climb but only marginally to levels more consistent with teleost fishes ($\sim 1 \text{ mmol L}^{-1}$; [Gerst and Thorson, 1977](#); [Tam et al., 2003](#); [Ip et al., 2009](#)). Even elevated environmental ammonia did not induce urea synthesis or excretion ([Ip et al., 2003](#)). There has been no work on early life stages in *Potamotrygon* to determine if the OUC is active during embryogenesis as in early life stages of many teleost fishes (reviewed by [Wright and Fyhn, 2001](#)), but it would be interesting to know whether the CPS III gene has been lost or is just turned down in mature fish.

5. CONCLUDING REMARKS

Biologists have explored many aspects of iono- and osmoregulation, as well as acid–base balance in elasmobranch fishes, but these discoveries still lag behind our comparable knowledge of teleost fishes. Studies of extant elasmobranch species are highly valuable because they can lead to broader insights into how early vertebrates evolved structures and functions to cope with water and solute balance. There is much to learn! For instance, it has been ~ 85 years since Homer [Smith \(1931b\)](#) first reported that in marine elasmobranchs urea is reabsorbed in the kidney to prevent loss. Five decades later [Lacy et al. \(1985\)](#) meticulously described the intricate and complex structure of the marine elasmobranch nephron, trying to link a possible mechanism for urea reabsorption with the unique renal morphology using “computer-assisted reconstruction.” More recently (1999–2008) several labs have identified and done some preliminary characterization of shark and skate urea transport proteins in the kidney. Do we now understand how the kidney reabsorbs urea and releases more with environmental salinity dilution? No, but this is an area full of potential for ambitious young researchers. There are also exciting changes in our understanding of ammonia transport pathways in teleost fishes, so studies on elasmobranchs may provide contrasting models because ammonia is scavenged

(elasmobranchs) rather than eliminated as a waste (teleosts). Moreover, ammonia transport is thought to be associated with H^+ secreting or exchanging transporters (e.g., NHE, H^+ -ATPase), and how these complexes operate in the gills, kidney, rectal gland, and gut are not understood in elasmobranch fishes. There has been new interest in how the gut is involved in acid–base, ion, and fluid balance. Further work is necessary to understand the link between regional heterogeneity in the gut and the potential multifunctional roles. There have been very few studies on the obligate freshwater Potomotrygonid stingrays to understand how ions and acid–base balance are regulated across different epithelial surfaces, including the degenerate rectal gland. To answer these and other questions, researchers need field stations. Much of the early foundational work on marine elasmobranchs, including the exceptional work of Homer Smith, was performed at the renowned Mount Desert Island Marine Biological Laboratory in Salisbury Cove, Maine, USA. Many of the recent studies over the last two decades have taken place on the west coast of Vancouver Island, British Columbia, Canada at the Bamfield Marine Sciences Centre. Having access to healthy elasmobranch populations and excellent physiological facilities for physiological work will be critical in understanding the physiology of these creatures in our changing oceans.

ACKNOWLEDGMENTS

Elasmobranch research in our laboratories, as well as the cost of preparing this review, was supported by NSERC (Canada) Discovery Grants to PAW and CMW. We thank Gary Anderson, Carol Bucking, Alex Clifford, Greg Goss, Jim Ballantyne, Jürgen Pollerspöck and Michele Nawata for helpful communication. We also thank Alexis Platek and Lori Ferguson for skilled typographical assistance and Sunita Nadella and Ian Smith for their wonderful artwork.

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FEEDING AND DIGESTION IN ELASMOBRANCHS: TYING DIET AND PHYSIOLOGY TOGETHER

CAROL BUCKING

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Examining the diet of elasmobranchs provides important ecological and physiological information. There are several techniques available for determining the diet of elasmobranchs including stomach content analysis, stable isotope analysis, and fatty acid analysis. Each technique has its own advantages and drawbacks and the aim of the study will determine the choice of technique, along with other technical considerations. Furthermore, diet preference appears to be dependent on both intrinsic and extrinsic factors that can provide insight into elasmobranch physiology. In addition, the diet may ultimately determine the physiology of the gastrointestinal tract itself, from overall activity level to enzyme expression. Anatomically, the elasmobranch gastrointestinal tract consists of several discrete structures and associated organs. Each organ has distinctive functions in digestion and

elasmobranchs possess several unique organs not found in other species. The digestion of consumed food proceeds through an assortment of digestive enzymes and secretions. The secretion of the enzymes is most likely dependent on the diet consumed, while activity levels appear enhanced in elasmobranchs compared to other aquatic ectotherms. Overall, elasmobranchs occupy a number of ecological niches and their species-specific physiology is suited to this spectrum of demands. Future work is needed to expand our knowledge of how the consumption of a variety of diets affects the gastrointestinal tract and elasmobranch physiology.

1. INTRODUCTION

Knowledge of the feeding habits of elasmobranchs has been used for a number of different aims: from studying the natural history and evolution of sharks, to modeling predator–prey interactions in marine ecosystems, to estimating the impact of feeding on commercially valuable prey. Ecologically, dietary information is crucial to assess the role of predators in community structure and dynamics, as well as a larger understanding of the ecosystem that the animals inhabit. Physiologically, as diet compositions show from where animals derive their sustenance, dietary information is crucial to understanding the homeostatic processes occurring in animals, both within the gastrointestinal tract (GIT) as well as throughout the body. Understanding what elasmobranchs eat, how often they eat, and whether their diet changes, will allow a better understanding of the physiology of the GIT observed in these species, as it has in teleosts (e.g., [Buddington et al., 1987](#)). While the ability of an elasmobranch to consume a wide array of prey species is a challenge in feeding studies, it does suggest an inherent plasticity or flexibility in GIT function. Interestingly, elasmobranchs possess a relatively anatomically uniform GIT across species despite a broad range in dietary preferences and occupation of ecological niches and trophic levels.

[Fig. 6.1](#) shows a simplified breakdown of possible feeding and digestion scenarios for sharks. First, the type of prey consumed may determine the type of feeding approach that is used, either continuous feeding (i) or intermittent feeding (ii). Subsequently, the type of feeding approach may have implications on the digestive processing in these animals ([Fig. 6.1](#)). For example, linear (e.g., [Cortés and Gruber, 1990, 1994](#)) and exponential (e.g., [Sims et al., 1996](#)) gastric evacuation rates have been observed in several types of elasmobranchs and could reflect continuous and intermittent feeding respectively ([Fig. 6.1](#)). Additionally, in order to digest different prey items efficiently, the type of enzymes expressed (and/or their

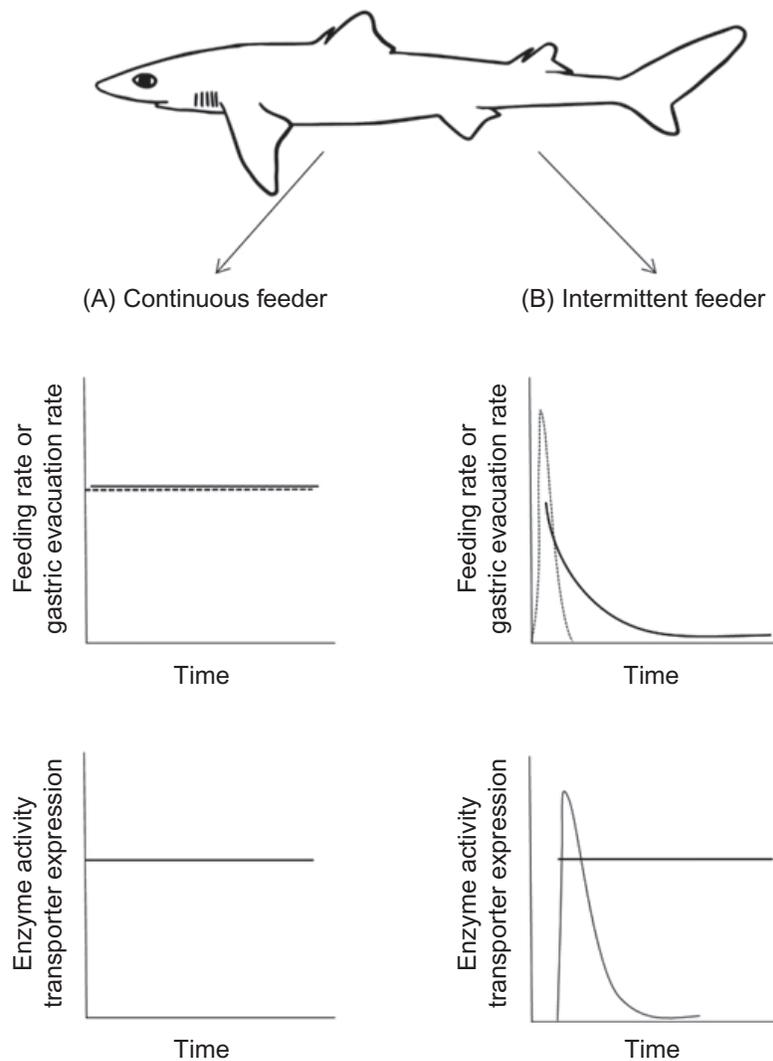


Figure 6.1. Potential elasmobranch feeding ecology and subsequent gastrointestinal tract physiology. Animals may either (A) continuously consume food (dashed line) and evacuate digesta from the stomach (solid line) or (B) consume individual meals infrequently (dotted line) and evacuate digesta from the stomach in an exponential rate (solid line). The consequences of the feeding strategy on the gastrointestinal tract physiology (for example enzyme activities, transporter expression, etc.) could reflect meal consumption frequency.

activity level) in the GIT may depend on both the length of time available for digestion, as well as the type of nutrients to be catabolized (Fig. 6.1). There is some evidence that continuous feeders maintain their GIT in a state that is prepared to digest food, whereas intermittent feeders may up- and down-regulate the GIT in response to feeding (e.g., Papastamatiou and Lowe, 2004, 2005; Papastamatiou, et al., 2007). Although not yet shown in

elasmobranchs, there is evidence that teleost fish are able to move between the categories depicted in Fig. 6.1. Hence, prey choice, feeding strategy, and digestive processing could affect GIT physiology in terms of molecular and cellular responses to feeding in order to optimize the digestion process (e.g., Buddington et al., 1987). Therefore, to understand the physiology of the GIT, it is important to know the type of food ingested and the frequency of feeding, as well as any changes in both throughout the life history of sharks.

2. FEEDING HABITS OF ELASMOBRANCHS

Optimal foraging theory (OFT) suggests that predators select the most energetically profitable prey item to maximize fitness (Pyke, 1984; Parker and Smith, 1990). Essentially, OFT predicts that animals maximize energy coming in and minimize energy going out, which results in increased growth, and eventually translates into reproductive success. To use OFT to examine elasmobranch physiology, we must first examine what elasmobranchs are consuming. For most terrestrial animals, simple observation of prey selection can estimate diet composition. However, direct observation of feeding behavior of elasmobranchs presents practical and technical limitations precluding this approach. Traditionally, researchers have identified prey items by examining the contents of GIT, either through morphological or molecular detection. While this approach yields important information, it is just a snapshot in time. Recent approaches, including stable isotope analysis and quantitative fatty acid analysis, rely on the persistent biochemical signature of long-term feeding history.

2.1. Techniques for Studying Elasmobranch Diets

2.1.1. STOMACH CONTENT ANALYSIS

Stomach content analysis (SCA) involves terminal sampling of the stomach and subsequent identification of items found therein. Morphological identification of stomach contents is the most standard method found in the literature. In this case, key anatomical traits are used to assess collected stomach contents and identification of the exact prey species is based on observed, defined morphological characteristics (e.g., Barnett et al., 2010). This is the most common approach found throughout literature and has provided most of the knowledge that we possess about elasmobranch diets. Technological advances have made possible the identification of stomach contents based on DNA analysis. This involves the development of universal primers that amplify the DNA of a wide range

of taxa. These primers are then used against the pooled DNA of all prey collected from the stomach of sampled animals. Subsequently, the observed prey DNA sequences are referenced to known sequences, identifying the species present in the stomach. More specific primers can be developed that are focused on specific groups (i.e., invertebrates vs. vertebrates or a specific class of animals; e.g., Deagle et al., 2007; Valentini et al., 2008), which requires previous knowledge of the prey species likely to be encountered. Using this approach with elasmobranchs has resulted in a 90–100% accuracy rate in prey species identification (Barnett et al., 2010; Sigler et al., 2006; Dunn et al., 2010); however, it is not currently a common approach.

Both approaches have advantages and disadvantages. The simplistic, inexpensive nature and straightforwardness of morphological identification are advantages of the approach (Table 6.1). Rapid identification carried out at the site of collection also obviates sample storage and transport concerns (Table 6.1). Beyond prey identification, morphological identification can also determine the relative proportions of prey types (Table 6.1; see Section 2.1.1.1). However, accurate identification of prey species based on morphology requires extensive expertise in aquatic animal morphology, from zooplankton to mammals, especially when omnivorous predators are examined (Table 6.1). Additionally, this approach often fails to achieve species-level identification, as key diagnostic features may be lost as tissues are broken down through mechanical and chemical digestion (Table 6.1; e.g., Barnett et al., 2010). A lack of hard remains from prey and/or soft bodied prey also may reduce the ability to identify species in stomach contents (e.g., Reñones et al., 2002), and evaluation of diet composition based on the percentage occurrence of prey in the stomach may be biased by differential rates of prey digestion (Table 6.1; e.g., Berg, 1979).

The primary advantage of a DNA-based approach is an improved taxonomic resolution (Table 6.1). Indeed previous studies show that prey DNA identification provides anywhere from a 25–100% increase in specific prey species identification relative to morphological approaches (Barnett et al., 2010; Sigler et al., 2006; Dunn et al., 2010). Another advantage of DNA identification over morphology-based methods is an increase in speed of data collection and generation (Table 6.1; Dunn et al., 2010). While offering a possible improvement over morphological approaches in the level of prey identification, a few disadvantages are specific to DNA approaches. This technique depends on the ability to match the prey DNA sequences against known sequences, which depends on the completeness of DNA databases (Table 6.1). Another disadvantage to this approach is that it requires relatively expensive tissue-specific extraction and analysis (Table 6.1; Deagle and Tollit, 2007). Recent advances in high-throughput sequencing techniques, which allow DNA identification of numerous taxa in large

Table 6.1
Advantages and disadvantages of various techniques for determining the diet consumed by elasmobranchs

| Technique | Advantage | Disadvantage |
|---|---|--|
| Stomach content analysis | | |
| 1. Morphological Identification | Inexpensive | Requires extensive expertise in marine animal morphology |
| | Rapid | Identifying characteristics may be affected by digestion |
| | Does not require advanced technology | Difficult to identify prey that lack hard remains |
| | Can account for relative proportions as long as recently consumed | Differential rates of prey digestion will create bias |
| | | Only identifies recently consumed items |
| 2. Molecular Identification | Highly accurate for specific prey identification | Relies on complete DNA database |
| | Rapid data collection and preservation | Can be expensive |
| | | Does not account for relative proportion of consumed items Only identifies recently consumed items |
| Stable isotope analysis | | |
| | Can reveal long and short term dietary sources | Low taxonomic resolution |
| | Can reveal geographic information | Rapid dietary shifts can create unreliable information due to slow tissue incorporation |
| | Can reveal ontogenetic dietary shifts | Unknown influence on signature by physiology |
| | Does not have to be invasive or destructive and only requires small tissue sample | Lipid interference |
| | | Accurate quantification via modeling is dependent on parameters that are often unknown for elasmobranchs |
| Quantitative fatty acid signature analysis | | |
| | Does not have to be invasive or destructive and only requires small tissue sample | Limited experimental knowledge of fatty acid incorporation in elasmobranchs |
| | May reveal more species-specific level of identification over SIA | |
| | Can reveal long and short term dietary sources | |

numbers (reviewed by [Murray et al., 2011](#)), may further increase the speed of the technique, but also the cost of the approach ([Table 6.1](#)). Finally, it is difficult to determine the amount of a prey consumed based on DNA, and this approach is more reflective of presence/absence information ([Table 6.1](#)).

A shared disadvantage between the two approaches is that SCA only assess recently consumed prey, providing no information on the long-term dietary preferences of animals, or ontogenetic or seasonal shifts in diet ([Table 6.1](#)). Cautious interpretation of these studies is required, as diet appears to shift with age, season, animal size, and geographic location, as described shortly.

2.1.1.1. Calculating dietary importance. There is a variety of approaches in quantitatively analyzing the diet based on collected stomach content prey identifications, each with their own advantages. More traditional, basic measurements of counts of individual prey items (numerical quantification of prey items identified in an individual stomach), frequency of occurrence (i.e., the proportion of stomachs containing a specific prey category), and prey volume (weight of individual prey items in a stomach) each reveal specific aspects of dietary information. Prey item counts are representative of immediate feeding behavior, while frequency of occurrence of prey observed across animals sampled reveals population-wide dietary habits, and volume or weight measurements reveals nutritional value of prey consumed. As a more accurate representation of the importance of individual prey items to the diet of predators, [Cortés \(1997\)](#) proposed a composite measure [the Index of Relative Importance (%IRI)], which incorporates the frequency of occurrence, the volume of prey consumed, and the numerical abundance of individual prey items. However, the feeding approach (continuous or intermittent), prey preference, prey size, and gastric evacuation rate all influence the performance of both basic and composition indices of diet analysis ([Ahlbeck et al., 2012](#)). Indeed, the analysis of continuously feeding predators and intermittently feeders ([Fig. 6.1](#)) showed biases in each analytical approach for estimating dietary importance, especially when examining specialized piscivores ([Ahlbeck et al., 2012](#)).

2.1.2. STABLE ISOTOPE ANALYSIS

Stable isotope analysis (SIA) is commonly employed to infer diet and trophic relationships within ecosystems and to reconstruct animal diets. SIA involves measuring ratios of heavier and lighter isotopes in animal tissue, and comparing it to the ratio of the particular isotope in an international standard (reviewed by [Post, 2002](#); [Martínez del Rio and Wolf, 2005](#)). Generated mathematical models then predict which prey contributed to the observed isotope signature in the predator.

However, observed isotope signatures are heavily dependent on the physiology of the organism and often do not directly reflect those seen in prey. These effects are dependent on both intrinsic and extrinsic factors. Firstly, distinct metabolic pathways determine differential isotope incorporation rates (Martínez del Rio and Wolf, 2005; Ben-David and Flaherty, 2012), a process that is dependent on specific enzyme activities. For example, the oxidation of pyruvate to acetyl coenzyme A affects carbon isotopes incorporation (Martínez del Rio and Wolf, 2005), and elasmobranch tissues or animals with increased pyruvate oxidation (e.g., Treberg et al., 2003) may display altered carbon isotope signatures. Other physiological factors such as animal size, age, stress, growth rate, and nutritional status can affect isotope incorporation rates (Sweeting et al., 2007a,b; Trudel et al., 2011; Weidel et al., 2011) in possibly tissue-specific manners (Caut et al., 2009; Martínez del Rio et al., 2009). Finally, the nitrogen excretion strategy employed by the predator (Minagawa and Wada, 1984; Vanderklift and Ponsard, 2003) can affect isotope signatures. Elasmobranchs are ureolytic, relying on a modified enzyme pathway to produce urea for osmoregulation (Ballantyne, 1997; Hazon et al., 2003). The resulting high urea levels in elasmobranch tissues require a modification of the SIA technique to avoid biasing the findings (Kim and Koch, 2012). External environmental factors such as temperature (Logan and Lutcavage, 2010; Bosley et al., 2002; Trudel et al., 2010) affect isotope signatures as well. Finally, though there are few euryhaline species of elasmobranchs (Wosnick and Freire, 2013), analysis of their diet would require consideration of the potential impact of changes in salinity, which is known to affect SIA (Caut et al., 2009).

SIA has been increasingly used in studies on elasmobranchs (e.g., Papastamatiou et al., 2010; Hussey et al., 2010b; Borrell et al., 2010, 2011; Matich et al., 2011; Speed et al., 2012; Kim et al., 2012a,b) to reveal important information about trophic level position within communities. These trophic levels often match those predicted by SCA (e.g., Cortés, 1999), which reveals the potential to replace this older approach. However, caution is needed because of a poor understanding of isotope incorporation in elasmobranchs (Hussey et al., 2010a) as well as a lack of baseline data (Post, 2002). Indeed, to date there have been few experimental studies to examine elasmobranch-specific isotope incorporation factors (Hussey et al., 2010b; Kim et al., 2012a,b). Existing evidence supports the use of elasmobranch-specific incorporation factors as rates of isotopes were slower (Kim et al., 2012b) than those observed in other aquatic ectotherms (MacAvoy et al., 2006; MacNeil et al., 2006; Logan and Lutcavage, 2010). Additionally, observed incorporation rates varied between tissues and individuals (Kim et al., 2012b). These results suggest that to create accurate

mathematical modeling of isotope incorporation in elasmobranchs, more research is needed on these animals.

SIA has several advantages over SCA. Isotopic ratios are representative of the ratios present at the time of tissue synthesis (Hobson and Clark, 1992) and depending on tissue chosen, SIA can be used to study the temporal variation of diet and habitat use in animals by exploiting tissues with different turnover rates (Table 6.1; Dalerum and Angerbjörn, 2005). Comparing tissues with varying rates of regeneration could offer an opportunity to generate information about dietary shifts throughout the animal's life history, and to construct migratory maps of animals without the need to recapture them later (Table 6.1; e.g., Sweeting et al., 2005; Martínez del Rio and Carleton, 2012). An interesting aspect to SIA is comparing the signatures of animals at various ages, thus revealing ontogenetic dietary shifts with growth (Borrell et al., 2011; Speed et al., 2012) or variability in migration and residency patterns that result in dietary shifts (Papastamatiou et al., 2010). The technique also offers a nondestructive method of tissue sampling (Table 6.1; e.g., blood collection, scale or teeth analysis, muscle biopsy).

Disadvantages of SIA in elasmobranchs have implications for the utility of the approach. There is low taxonomic resolution with this technique and often trophic level estimations are the only result of SIA (Table 6.1; e.g., Matich et al., 2011; Speed et al., 2012). As well, elasmobranchs can have a slow stable isotope turnover rate depending on which tissue is examined (Logan and Lutcavage, 2010). Hence, measurements may miss recent dietary shifts and studying stable isotope incorporation in the lab is difficult (Table 6.1). As outlined above, the physiology of the animal influences isotope signatures and without experimental support, extrapolations from other species may adversely affect the formed conclusions. For elasmobranch study specifically, lipids in particular interfere with isotope measurements (Post et al., 2007; Murry et al., 2006) and therefore must be chemically extracted from the tissue before analysis (Sweeting et al., 2006; Post et al., 2007; Logan et al., 2008). Some shark tissues such as the liver, which are particularly rich in lipids (e.g., Pethybridge et al., 2014), exhibit a known analytical bias when measuring stable isotopes (Table 6.1; Hussey et al., 2010b; Kim and Koch, 2012).

In summary, there are a number of parameters that must be chosen to accurately reflect variables, such as predator physiology or tissue composition (Martínez del Rio and Wolf, 2005; Moore and Semmens, 2008; Parnell et al., 2010; Kim and Koch, 2012), when building a model for SIA. Without experimental supporting evidence, estimates are used to construct these mathematical models (Table 6.1; Phillips and Gregg, 2001, 2003; Moore and Semmens, 2008) and they may not accurately predict diet composition.

There is some evidence that SIA and SCA studies do not agree (i.e., [Kim et al., 2012b](#) vs [Cortés, 1999](#)) and these differences may result from time of sampling, tissue choice, and/or incorrect models or isotope incorporation factors calculated for SIA.

2.1.3. QUANTITATIVE FATTY ACID SIGNATURE ANALYSIS

A final approach to determine prey selection in elasmobranchs is the use of fatty acid (FA) signatures ([Iverson et al., 2004](#); [Iverson, 2009](#)). FAs are the main component of most lipids and during digestion are released from ingested lipid molecules and are often absorbed into the circulation and then into cells intact ([Iverson et al., 2004](#)). Additionally, as marine vertebrates do not synthesize n-3 and n-6 long-chain polyunsaturated fatty acids (PUFAs), integration through the diet is required and thus can act as biochemical indicators of food webs ([Tocher and Ghioni, 1999](#); [Dalsgaard et al., 2003](#); [Iverson et al., 2004](#); [Thiemann et al., 2008](#)). This has led to the use of quantitative fatty acid signature analysis (QFASA) to investigate the lipid composition of predators and to reveal dietary prey composition, much like SIA.

In most marine vertebrates, lipids are stored in the adipose tissue typically located in muscle or blubber ([Budge et al., 2011](#)). However, elasmobranchs oxidize FAs obtained from lipids in the liver ([Moyes et al., 1990](#); [Ballantyne, 1997](#)). Interestingly, though elasmobranchs typically lack adipose tissue in their muscle, several studies have shown the presence of dietary FAs in this tissue ([Pethybridge et al., 2010, 2011, 2014](#)). As with SIA, the basis of this technique requires a knowledge of FA signatures in the prey, an understanding of how FA are incorporated into tissues during digestion and assimilation, predator tissue sampling, and a mathematical model to analyze the predator FA signature into the appropriate prey species composition. QFASAs have recently become more popular in chondrichthyan research ([Pethybridge et al., 2010, 2011, 2014](#); [Beckmann et al., 2013, 2014](#)). Importantly, initial controlled laboratory research has shown that both liver and muscle FA profiles are indicative of dietary shifts ([Beckmann et al., 2014](#)).

There are several advantages of QFASA. Both, SIA and QFASA have the advantage of only requiring a small amount of tissue for analysis, obtained as a biopsy from living animals ([Table 6.1](#)). Like SIA, it might be possible to identify changes in diet by examining tissues with differential incorporation rates ([Table 6.1](#); [Beckmann et al., 2013, 2014](#)). Additionally, evidence suggests that QFASA may reveal more species-level specificity compared to higher trophic level identification through SIA ([Table 6.1](#); [Budge et al., 2002](#)). Indeed, identification of unique FA patterns in marine

fish and invertebrates potentially allows for individual prey species identification (e.g., Iverson et al., 2002; Budge et al., 2002).

However, there has been limited experimental investigation into how prey selection influences the FA profiles of different predator tissues in a controlled environment (Table 6.1). There is also limited information on how the physiology of the predator affects FA incorporation in a tissue specific manner, as is the case with SIA (Table 6.1). For example, the FA profiles of Port Jackson sharks (*Heterodontus portusjacksoni*) fed different diets were indistinguishable in liver but were apparent in the muscle (Beckmann et al., 2013). This may reflect tissue-specific differences in processing lipids, or that the timescale of that study was not adequate to observe the integration of the FA into liver tissue (Beckmann et al., 2014). Future experimentation is needed to investigate temporal FA integration into various tissues, under a variety of environmental constraints, and across life stages. Without these experimental controls, conclusions reached using QFASA may have to be viewed with caution.

Ultimately, combining complimentary biochemical methods such as QFASA and SIA, possibly along with mechanical methods such as SCA, is likely to give broad, useful modeling information. Using these techniques synergistically creates resolutions in consumed prey across varying time-scales, and provides a picture of both current and historical dietary information (Couturier et al., 2013; Connan et al., 2014). QFASA and SIA together also provide important behavioral data, which in turn provides important physiological data, especially if the animal encounters variable environments (Couturier et al., 2013).

2.2. Elasmobranch Diet Composition and Dietary Shifts

The combination of techniques discussed above reveals surprising flexibility in some species and constraints in others. The most familiar elasmobranchs are the apex predators, but elasmobranchs collectively include both generalists and specialists, live in a wide range of habitats, and consume diverse prey (Fig. 6.2). Unfortunately, a number of elasmobranch species do not fit the strict definitions of generalists and specialists. Instead, they appear to exist on a continuum between generalists and specialists both in regards to diet and/or niche occupation (Fig. 6.2; Munroe et al., 2014). To illustrate the problem of categorizing elasmobranchs, a shark can exhibit a far-reaching, global habitat range but be restricted to a narrow range of prey item selection such as the whale (*Rhincodon typus*) or basking (*Cetorhinus maximus*) sharks (Fig. 6.1; Munroe et al., 2014). In contrast, there are sharks that have narrow habitat ranges and wide prey selectivity such as the black tip reef shark (*Carcharhinus melanopterus*; Fig. 6.2;

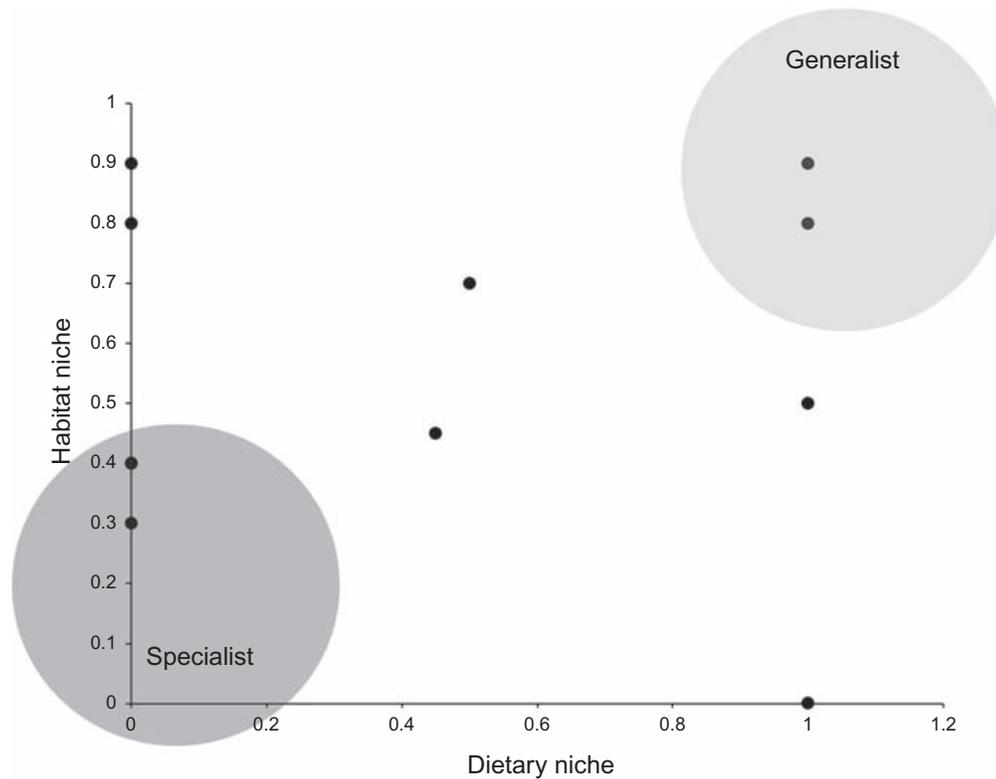


Figure 6.2. Dietary niche and corresponding habitat niches of several elasmobranch species. Dietary niche is defined as the total number of different classes of prey species consumed, with 0 being a single prey species class consumed and 1 being prey consumed from 11 different classes. Habitat niche is defined on a scale of global range with 0 being almost no movement of an individual from the birth location, and 1 being a capability of wide global movement of an individual away from the birth location. A specialist is defined as an individual with low range in habitat and number of species consumed, while a generalist is defined as have a large scope for habitat and diet change. Data points represent individual species of sharks. Data compiled from Cortés (1999), Belleggia et al. (2008), Hoffmayer et al. (2014), and Munroe et al. (2014). Example shark species in the figure include the dusky shark (*Carcharhinus obscurus*), whale shark (*Rhincodon typus*), great white shark (*Carcharodon carcharias*), tiger shark (*Galeocerdo cuvier*), bull shark (*Carcharhinus leucas*), white tipped reef shark (*Triaenodon obesus*), gray reef shark (*Carcharhinus amblyrhynchos*), black tip reef shark (*Carcharhinus melanopterus*), porbeagal shark (*Lamna nasus*), and broadnose skate (*Bathyraja brachyurops*).

Munroe et al., 2014). There are also a variety of feeding behaviors present in elasmobranchs, including selective (feeding on prey in a disproportionate manner to their availability) and opportunistic (feeding on prey in a proportionate manner to their availability) feeding behaviors. An elasmobranch can be characterized as an opportunistic specialist – where the narrow type of diet that is selected is the most available, while another can be a selective generalist – choosing a wide assortment of individual prey

items that may be proportionally rarer compared to other species in the area. As discussed later in the current chapter, there are physiological implications for dietary choice and specialization in regards to intestinal physiology, which reveals the need to define the paradigms used in elasmobranch biology.

In 1999, Cortés compiled a list of sharks, their diet composition, and inferred trophic levels. A quick scan of the compiled list provides examples of loosely defined specialists and generalists, while trophic levels range from 3.1 to 4.7. Compilations on skates (Ebert and Bizzarro, 2009) and rays (Jacobsen and Bennett, 2013) likewise revealed diverse foraging strategies, with specialists and generalists present and similar trophic levels occupied compared to sharks. Ultimately, these studies show the failings of broadly characterizing elasmobranchs as apex predators, and illustrate the breadth of prey and foraging strategies present (Fig. 6.2). Understanding resource use of elasmobranchs provides information critical for successful management solutions for declining populations, as well as for ecosystem protection through understanding community structure. Physiologists can use information of dietary preferences and habitat use (depth, temperature, salinity, etc.) to predict and investigate GIT physiology as discussed below.

2.2.1. CARNIVORES

The majority of elasmobranch species are classified as carnivores, and there is no record of any plankton or plant material in stomach contents being detected. These predators only consume other animals such as cephalopods, crustaceans, fish, elasmobranchs, or aquatic mammals and birds (Cortés, 1999; Ebert and Bizzarro, 2009; Wetherbee et al., 2012; Jacobsen and Bennett, 2013). Cephalopods are a common prey item of many elasmobranch species. For example, only 21 species of shark failed to consume cephalopods of the 149 species examined by Cortés (1999). A few species of carnivorous elasmobranchs appear to specialize on certain prey items, defined here as >80% of prey items consumed. For example, *Mustelus californicus* preys almost exclusively on crustaceans, and 19% sharks species noted in Cortés (1999) specialize in preying on teleost fish species. There are also generalist elasmobranch predators that consume a variety of prey animal resources (Wetherbee et al., 2012); however, they may not conform to the strict definition of generalist and may have relatively narrow ecological niche occupancies (Fig. 6.2). It is not clear if this feeding approach reflects an opportunistic feeding pattern as prey densities are rarely co-examined, but the breadth of prey consumed certainly suggests a flexibility in prey capture and digestion.

2.2.2. PLANKTIVORES

Filter-feeding of plankton is found in three separate orders: the Myliobatiformes, the Lamniformes, and the Orectolobiformes. Mobulidae (comprised of two recognized species of manta rays and nine species of devil rays) are zooplanktivorous elasmobranchs as indicated by studies using SCA (Notarbartolo-di-Sciara, 1988; Celona, 2004) and SIA (Sampson et al., 2010; Borrell et al., 2010). These species feed on a variety of euphausiids and mysids, with some evidence that they have the flexibility to shift diets between the two depending on the dominant prey in the particular feeding location suggesting they are opportunistic predators (SCA; Notarbartolo-di-Sciara, 1988). However, there is evidence that at least one ray species (*Mobulae japonica*) specializes in a single euphausiid species (SCA and SIA; Sampson et al., 2010). Several other elasmobranch species are planktivores including whale sharks (Gudger, 1941; Motta et al., 2010), basking sharks (Sims, 1999), and the megamouth shark (*Megachasma pelagios*; Nakaya et al., 2008). There is evidence that basking sharks may be specialist feeders, optimally foraging on *Calanus helgolandicus* (Sims and Merrett, 1997). These large planktivores are generally thought to be continuous feeders (Fig. 6.1), feeding regularly and often to meet their metabolic demands with such small prey, although this is mostly speculative knowledge based on animal tracking data using global positioning system (GPS) tags, and correlations with zooplankton availabilities (Anderson et al., 2011; Braun et al., 2014). Sims (2008) estimated that a basking shark consumes 30 kg d⁻¹ of plankton.

2.2.3. OMNIVORES

Only a few species of sharks can be classified as true omnivores, consuming prey across phyla from plants to invertebrates to vertebrates (Cortés, 1999). Bethea et al. (2007) detected large quantities of plant material in the stomach of bonnethead sharks (*Sphyrna tiburo*) representing between 15 and 62% IRI. No experiments were conducted to reveal if the bonnethead shark GIT contains the enzymes required for the digestion of plant material, such as cellulases, and the role of the plant material detected in these animals is not clear.

2.2.4. DIETARY SHIFTS

Shifts in prey species found in the GIT represent a challenge for elasmobranch feeding studies, but also produce good physiological models to study on the effect of diet on GIT function. For example, when a lemon shark (*Negaprion brevirostris*) shifts from crustaceans to fish as the main prey source (Newman et al., 2012), this likely requires a compensation such

as differential enzyme activity patterns in the GIT, as is seen in teleosts (e.g., Chakrabarti et al., 1995; Hidalgo et al., 1999; Drewe et al., 2004). Certainly, chitinase activity would likely diminish with a reduced need to extract nutrients from chitin exoskeletons.

Intrinsic factors such as individual variation and ontological influences can cause dietary shifts. Ontogenetic shifts in elasmobranch diets are commonly observed (e.g., Bethea et al., 2007; Polo Silva et al., 2013; Newman et al., 2012) and are often attributed to the ability to catch larger prey increasing with increasing body size (e.g., Lucifora et al., 2009; Newman et al., 2012) which may increase foraging profitability. This energetic benefit of feeding on large prey is consistent with studies based on OFT of other fish species (Werner and Gilliam, 1984). Baremore et al. (2010) showed that Atlantic angel sharks (*Squatina dumeril*) exhibited prey size selection in accordance with their own gape width. As these sharks consume prey whole (Fouts and Nelson, 1999), this represents the functional limitations of prey size for consumption (Gill, 2003). Ontological shifts can also be caused by changes in habitat occupation and encountered prey communities (e.g., Bethea et al., 2007, 2011; Barbini and Lucifora, 2011). Ontological shifts in diet can change a more generalist juvenile animal (with a broad dietary niche) to a more specialized adult animal (with a smaller dietary niche; Belleggia et al., 2008), further complicating the precise classification of animals (Fig. 6.2).

Extrinsic factors such as geographic and seasonal changes in prey can also create variation in prey consumed by elasmobranchs. For example, the diet of the spiny dogfish (*Squalus acanthias*) in New Zealand is primarily crustaceans (SCA: Hanchet, 1991) while spiny dogfish found in Patagonian waters primarily consume squid (SCA: Alonso et al., 2002), although this had changed dramatically from historical data where hake was the primary prey, illustrating the flexibility of the dogfish to exploit various prey sources. In contrast, spiny dogfish off the West Coast of Canada fed mainly on teleosts in the winter and invertebrates in the summer (Jones and Green, 1977). This regional specification in prey consumption has also been observed for the sandbar shark (*Carcharhinus plumbeus*; McElroy et al., 2006; Cliff et al., 1988; Medved et al., 1985). These differences most likely reflect differences in habitat associated with different geographic locations (e.g., seagrass beds vs. hard bottom substrates) as seen in the bonnethead shark (Bethea et al., 2007).

Ultimately, prey selection appears to depend on ontogeny, geographic location, season, and prey abundance and it is difficult to predict which factor, or combinations of factors, will be important for each species of elasmobranch. Belleggia et al. (2008) showed that ontological shifts in diet selection were present while differences in geographic location showed little

influence on prey selection in the broad nose skate (*Bathyraja brachyurops*). In contrast, the Australian weasel shark (*Hemigaleus australiensis*) showed a significant influence of ontogeny and geographic location (Taylor and Bennett, 2008) while the bonnethead shark showed ontological differences only in certain geographic locations (Bethea et al., 2007). The spotback skate (*Atlantoraja castelnaui*) showed changes in prey selection with ontogeny, shifting from decapods to teleosts and elasmobranchs with increasing size, seasonal and regional changes in prey abundance (Barbini and Lucifora, 2012).

Regardless, shifts in prey preference and size selection may have significant impacts on GIT physiology. Ontological shifts in prey, for example in the spiny dogfish moving from smaller to larger prey (Bowman et al., 1984), may allow movement between the suggested paradigms in Fig. 6.1. Finally, shifting from energetically lower value animals such as decapods to energetically rich prey such as teleosts, as happens in the spotback skate (Barbini and Lucifora, 2012), will present the intestine with higher nutrient loads, potentially affecting metabolic pathways, transporter expression, and gastric evacuation rates (Sibly, 1981).

2.3. Food Consumption Rates and Gastric Evacuation

OFT is based on the assumption that predators maximize their fitness by selecting their diet to reflect energetically profitable prey. OFT then provides the framework around which to form hypotheses and test theories about prey selection, behavior, and evolution (e.g., Pyke, 1984; Parker and Smith, 1990). OFT includes models of optimal prey digestion which suggest that consuming nutritionally low-quality food necessarily results in ingesting larger amounts of such food and/or developing larger GITs when contrasted with consuming higher quality foods (Sibly, 1981). Optimal prey digestion also predicts that an increasing quality of prey items will result in a decreased transit or digestion time (Sibly, 1981).

In Fig. 6.1, a paradigm is suggested where consumption rates and prey (or ration) size or type may reflect both prey consumed and the physiology of the GIT. Increased feeding rates or decreased ration size in elasmobranchs can increase the speed at which digesta or chyme travels through the GIT (e.g., Meyer and Holland, 2012). Indeed, gastric evacuation time was increased by 50% when meal size was decreased by 8-fold in the scalloped hammerhead shark (*Sphyrna lewini*; Bush and Holland, 2002). Sims et al. (1996) found an inverse correlation between time until appetite return and the gastric evacuation rate in the lesser spotted dogfish (*Scyliorhinus canicula*), which suggests that as GIT transit times decrease, the more frequently elasmobranchs will feed. The size of the prey items may also

influence gastric evacuation times in elasmobranchs, with larger prey taking longer to digest (Bush and Holland, 2002). Conversely, consuming smaller prey more often will also affect the gastric digestion pattern, increasing feeding frequency and/or digesta passage rates. Taken all together, this logically suggests elasmobranchs that feed on large prey or that ingest large quantities at once will feed less often (intermittent feeders), while those that feed on smaller prey or take smaller meal sizes will be more continuous feeders (Fig. 6.1).

In teleost fish, other factors affect GIT physiology and the rate of digesta processing. The size of the predator itself affects gastric evacuation times, with smaller teleosts evacuating the GIT at a higher relative rate ($\text{g digesta g body mass}^{-1} \text{ h}^{-1}$) than larger teleosts (e.g., reviewed by Bromley, 1994; Gillum et al., 2012). These findings appear variable and may reflect prey type and/or growth as a confounding factor (e.g., Dunbrack, 1988). There have been no studies conducted on elasmobranchs to determine if this correlation is likewise observed. Digestion of different prey species may occur at different rates in several teleost species and potentially elasmobranchs (Jackson et al., 1987). *In vivo* evidence suggesting that prey with hard exoskeletons take longer to evacuate and digest compared to soft-bodied prey support these *in vitro* studies (Jones, 1974; MacDonald et al., 1982; Bromley, 1991). This is of particular relevance to SCA as differential rates of prey digestion could bias conclusions about diet composition (Table 6.1; e.g., Berg, 1979). Crabs and octopi require a longer digestion time compared to teleost fish in sandbar sharks, increasing digestion time by 20 h (Medved et al., 1985; Medved et al., 1988). Selection of prey sizes may depend on stomach fullness and/or appetite as frillfin gobies (*Bathygobius soporator*) choose prey size inversely proportional to stomach fullness (Tomida et al., 2012). It is unknown if this is also true for elasmobranchs.

The anatomy of the GIT itself may affect gastric evacuation times. In teleost fish, species-specific evacuation times are thought to be partially attributed to narrow sphincters and intestines, restricting the passage of large undigested objects from the stomach (Edwards, 1971; Kionka and Windell, 1972) resulting in a slower evacuation and longer digestion time. A larger intestine diameter may represent an adaptation in increased nutrient assimilation (MacDonald et al., 1982) while increased intestine length may slow digestion times in many herbivores with efficient digestion (Sibly and Calow, 1986; Munoz and Ojeda, 2000; Buckle and Booth, 2009). Wetherbee and Gruber (1993) reported that digestion time in the carnivorous lemon shark was longer than that observed in similar carnivorous teleosts (Wetherbee and Gruber, 1990; Sims et al., 1996; Wetherbee et al., 1990). The protracted time of digesta retention may be related to the shorter intestine in sharks compared to

teleost fish (Section 3.4), which reflects an increase in time needed by sharks to extract nutrients (Sibly, 1981).

Another factor that affects gastric evacuation and digestion time is environmental temperature, inversely decreasing the time it takes to process a meal with increasing temperatures (e.g., Gillum et al., 2012). Additionally, bonnethead sharks show selection for increased ration size with decreasing latitude, suggesting that with increasing water temperatures the increased metabolic demand must be met by increasing energy consumed (Bethea et al., 2007).

More work is needed to address the hypothesized paradigm presented in Fig. 6.1, and to assess the similarity or differences of elasmobranchs with other ectotherms. Nevertheless, species-specific feeding ecology is likely to influence digestive physiology (Fig. 6.1; Secor and Diamond, 1998; Papastamatiou and Lowe, 2005). Feeding frequency is responsible for species-specific differences in the digestive physiology for a number of reptile species (Secor et al., 1994; Secor and Diamond, 1998), and it is possible that this will be true for elasmobranchs as well. Papastamatiou and Lowe (2004) proposed that continuous gastric acid secretion in elasmobranchs is a primitive mechanism to increase the speed of digestion causing a more rapid return of appetite and hence energy consumed (Wetherbee et al., 1990; Sims et al., 1996). In fact, Papastamatiou (2007) predicted that digestion time decreased by ~6 h in the leopard shark (*Triakis semifasciata*) when the stomach was already acidic prior to meal consumption. This may be advantageous for animals that consume resources that are unpredictably available and/or are opportunistic or continuous feeders. They further hypothesize that maintaining gastric acid secretion is energetically efficient compared to down-regulation and then subsequent resynthesis and expression of transporters, proteins etc. (Papastamatiou and Lowe, 2005), much like the state of readiness observed in the intestines of snakes that feed regularly (Secor et al., 1994). It would be interesting to test this hypothesis with other continuously feeding shark species such as the lemon, sandbar, and scalloped hammerhead (Medved et al., 1985; Cortés and Gruber, 1990; Bush and Holland, 2002). In contrast a sporadic feeder, such as has been suggested for the spiny dogfish (Jones and Green, 1977; Tanasichuk et al., 1991; Wood et al., 2005), may have GIT physiology closer resembling an intermittently feeding teleost or possibly a sporadically feeding reptile (e.g., Secor and Diamond, 1998) with a large scope for changes in intestinal physiology during feeding and fasting.

Feeding frequency as cautiously inferred from the proportion of animals caught with empty stomachs, suggests that bonnethead sharks feed rather continuously while animals like the blacktip shark (*Carcharhinus limbatus*), the finetooth shark (*Carcharhinus isodon*), the dogfish, and the spinner

shark (*Carcharhinus brevipinna*) are intermittent feeders (Jones and Green, 1977; Bethea et al., 2004, 2007). The infrequency of feeding may depend on diet consumed, which in turn may depend on the environment, or geographical region the animal inhabits (e.g., Bethea et al., 2007). Clearly, a number of factors affect gastric evacuation rates and little experimental evidence exists examining elasmobranchs specifically.

3. ELASMOBRANCH GASTROINTESTINAL TRACT ANATOMY

The basic function of the GIT is to provide an avenue for the ingestion, digestion, and absorption of food and energy through mechanical, chemical, and transport processes. A number of specific anatomical divisions in the GIT, namely the buccal cavity and pharynx, the esophagus, the stomach, and the intestine, are responsible for these processes. There may be associated organs with each of these divisions that may or not be functionally obligated to the process of digestion. The reader is encouraged to pursue the wide assortment of additional reviews on elasmobranch GIT anatomy (Fänge and Grove, 1979; Holmgren and Nilsson, 1999; Cortés et al., 2008).

Teleost fish occupy several ecological niches and trophic levels similar to elasmobranchs. Unlike elasmobranchs, teleost fish demonstrate a diversity in GIT anatomy (Kapoor et al., 1975; Fänge and Grove, 1979; Wilson and Castro, 2010), having evolved to suit the diet consumed as well as to serve a variety of other functions such as air breathing (e.g., Graham, 1997; Nelson, 2014) and osmoregulation (e.g., Grosell, 2007; Larsen et al., 2014). Numerous studies have investigated the anatomical adaptations of the teleost GIT to various diets (Wilson and Castro, 2010). Most studies reveal that while correlations between diet and anatomical structures are weak, correlations between functional adaptations (i.e., enzyme expression profiles) appear related to the nature of the prey consumed (e.g., Chakrabarti et al., 1995; Hidalgo et al., 1999; Drewe et al., 2004). Unlike teleost fish, which also display a range of dietary preferences, the gross anatomy of the GIT of elasmobranchs is not as reflectively diverse across species. There are several smaller, species-specific variances in the anatomy addressed below, but overall the GIT of elasmobranchs is similar across species.

3.1. The Buccal Cavity, Pharynx, and Associated Structures

The buccal cavity in elasmobranchs represents the entry to the GIT and is associated with the selection and seizure of prey. There are varieties

of morphological buccal adaptations in elasmobranchs that are representative of dietary specializations along with associated morphology of the feeding mechanisms (e.g., [Motta and Huber, 2012](#)). Subsequent studies examining the function of the feeding mechanisms reveal much about the prey targeted by the sharks as well as their feeding strategies (e.g., suction feeding, ram feeding, bite feeding) and an excellent review exists ([Motta and Huber, 2012](#)) on the functional morphology of the buccal cavity and its association with the rest of the feeding mechanism. The lining of the buccal cavity consists of modified placoid scales in the cow shark (*Heptanchus maculatus*; [Daniel, 1934](#)) and lacks glands present in higher vertebrates. The lining of the pharynx consists of stratified cuboidal cells interspersed with mucous glands ([Chatchavalvanich et al., 2006](#)). The basking shark pharyngeal epithelium is additionally marked with papillae that may aid in food particle capturing ([Matthews and Parker, 1950](#)).

The gills slits are located in the ventrolateral walls of the pharynx through which the respiratory water current reaches the gills for gas exchange. Supporting their adaptations to filter feeding, pharyngeal sieving of food particles has been noted in the Mobulidae ([Cortés et al., 2008](#); [Paig-Tran et al., 2013](#)). The Lamniformes (basking sharks and megamouth sharks) and Orectolobiformes (whale sharks) have particularly large and complex sieving plates for filtering and trapping food particles ([Cortés et al., 2008](#); [Paig-Tran et al., 2013](#)). Associated with the pharynx are the thymus and thyroid glands ([Luer et al., 1995](#)). The associated pharyngeal musculature is similar to that seen in teleost fish ([Mallatt, 1997](#)).

3.2. The Esophagus

The pharynx leads directly into the esophagus ([Fig. 6.3A and B](#)), often separated by a sphincter that is closed except when passing food. The cells found within the esophagus are typically stratified columnar epithelial cells with abundant mucous cells ([Fig. 6.4A](#); [Holmgren and Nilsson, 1999](#); [Chatchavalvanich et al., 2006](#)). Occasionally there are long, finger-like projections into the esophageal lumen ([Leake, 1975](#)). The purpose of these projections are not clear.

The Leydig organ is an important esophageal-associated organ in most species of elasmobranchs and constitutes two masses of tissue found along the dorsal and ventral portions of the esophagus ([Fig. 6.4A](#); [Mattisson and Fänge, 1982](#); [Li et al., 2013](#)). The role of this unique organ is to aid in the production of red blood cells (also carried out by the spleen as in most other vertebrates) and other lymphoid activities categorizing it as a lymphomyloid

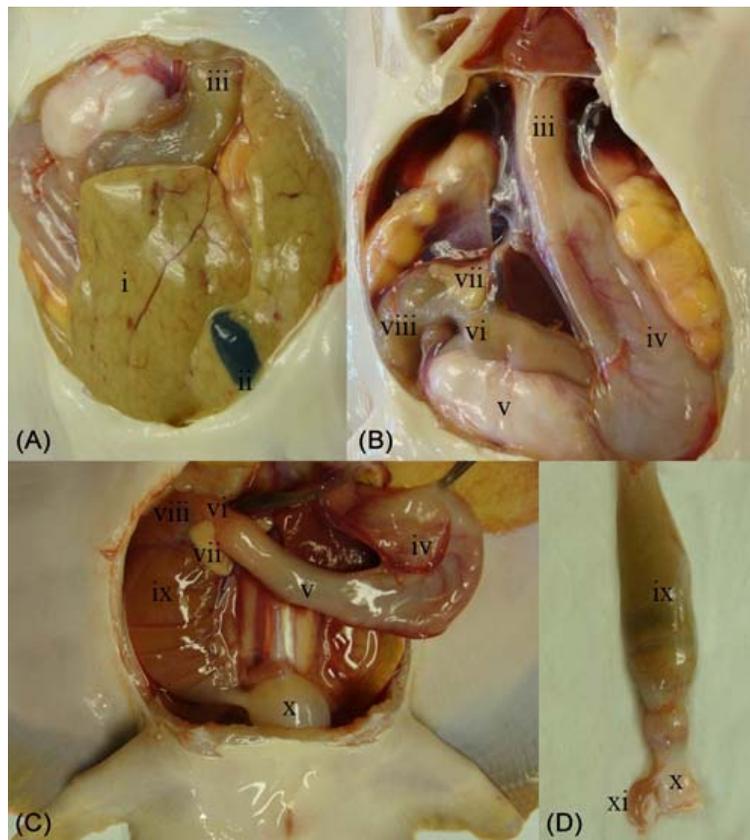


Figure 6.3. The gastrointestinal tract of the skate. (A) Ventral view of liver (i), gallbladder (ii), and esophagus (iii). (B) Ventral view of esophagus (iii; not pictured: Leydig Organ), cardiac stomach (iv), pyloric stomach (v), bursa entiana (vi), pancreas (vii), and anterior intestine (viii). (C) Ventral view of cardiac stomach (iv), pyloric stomach (v), bursa entiana (vi), pancreas (vii), and anterior intestine (viii), spiral intestine (ix), and colon (x). (D) View of spiral intestine (ix), colon (x) and rectal gland (xi) removed from animal. Photos provided by W.G. Anderson.

tissue (Mattisson and Fänge, 1982). According to Mattisson and Fänge (1982), the Leydig organ can be quite large – 1.6 kg of tissue in a cow shark (1.8 m in length) and 1.2 kg of tissue in a Greenland shark (*Somniosus microcephalus*; 2.9 m in length) and can account for 0.5% of total body weight. Early light microscopy and ultrastructural characterization has revealed a similarity between Leydig organ cells and those from the epigonal organ in elasmobranchs as well as those from the mammalian lymphomyeloid systems (Mattisson and Fänge, 1982). Recent investigations using molecular approaches have revealed that the Leydig organ appears to function in conjunction with the thymus and epigonal organs as the primary sites of lymphopoiesis (Anderson et al., 2004; Li et al., 2013).

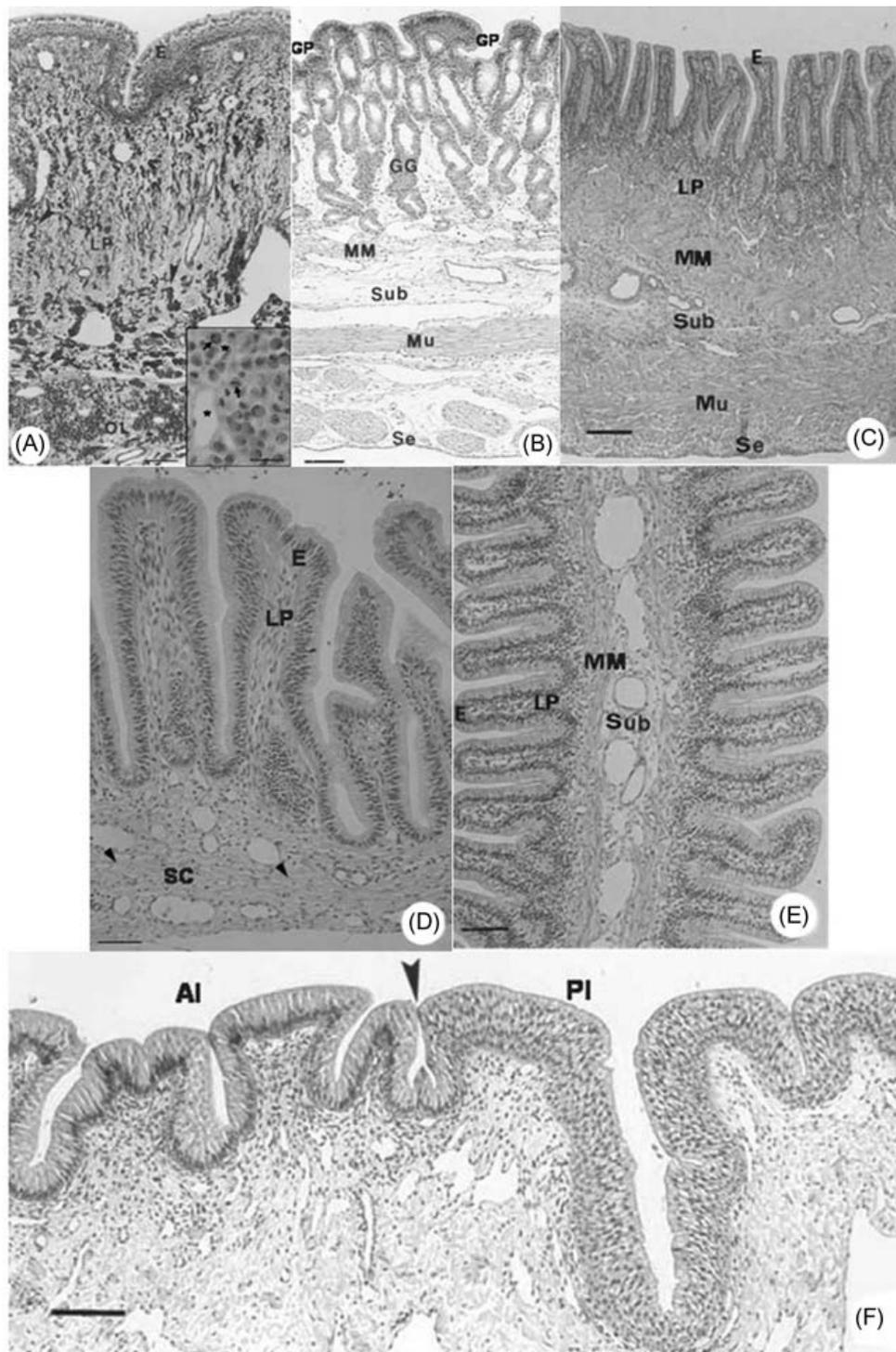


Figure 6.4. Histology of the gastrointestinal tract of the freshwater stingray (*Himantura signifier*). (A) Cross section of the esophagus epithelium (E) and lamina propria (LP). Collagen fibers are present in the LP (arrowhead). Also shown is the organ of Leydig (OL) embedded in the LP; inset shows OL as lymphomyeloid tissue with blast cells (arrows) and leucocytes (asterisk). Bar = 100 μ m. Inset: Bar = 25 μ m. (B) Cross-section of the cardiac stomach,

3.3. The Stomach

Lying posterior to the esophagus is the stomach. The stomach stores ingested food material and begins the initial chemical and enzymatic breakdown of food during digestion. Typically, it is characterized as a J-shaped or U-shaped organ in a species-specific pattern (Holmgren and Nilsson, 1999). The esophagus connects to the anterior, or descending portion of the stomach, which is known as the cardiac portion of the stomach (Fig. 6.3B). In contrast, the posterior, or ascending portion, is known as the pyloric stomach (Fig. 6.3B). The lining of the stomach consists of columnar cells with microvilli (Fig. 6.4B and C; Chatchavalvanich et al., 2006), which not only line the stomach but also make up the gastric glands interspersed in the stomach lining. Interestingly, histochemical characteristics of many of these columnar cells are indicative of absorptive cells (Chatchavalvanich et al., 2006). Columnar cells are also found in teleost stomachs (Grau et al., 1992) and whole animal evidence suggests the stomach is a site of absorption (Bucking and Wood, 2006, 2007). Recently, observed ion absorption in the stomach of the spiny dogfish (Liew et al., 2013) suggests this may be a shared phenomenon.

The gastric glands of elasmobranchs are the sites of digestive fluid secretions, namely HCl and pepsinogen, and are flask-like in shape and consist of a variety of cells (Fig. 6.4C). The current assumption is that lower vertebrates such as fish and sharks have a single type of secretory gland cell in the gastric gland – the oxynticopeptic cell (Fänge and Grove, 1979; Grabowski et al., 1995; Hamlett et al., 1996; Holmgren and Nilsson, 1999). This cell is responsible for the secretion of both HCl and pepsinogen for digestion. This is in contrast with higher vertebrates such as mammals that have separate, distinct cells for each: parietal cells for HCl secretion and chief cells for pepsinogen secretion. This view has been called into question after several studies on elasmobranchs revealed the potential presence of separate parietal and chief cell equivalents in the sixgill shark (*Hexanchus*

showing gastric pits (GP) and gastric glands (GG) in the mucosa. Muscularis mucosae (MM), submucosa (Sub), muscularis (Mu), serosa (Se) also shown. Bar = 100 μ m. (C) Cross-section of the pyloric stomach epithelium showing the LP and the thin stratum compactum (SC) (the upper limit of the SC is marked by arrowheads). Bar = 40 μ m. Note the lack of gastric glands. (D) Cross section of the anterior intestine epithelium. Bar = 100 μ m. Note the numerous mucosal folds. (E) Cross section of the spiral intestine, showing the spiral valve in detail. Bar = 50 μ m. (F) Longitudinal section of the anterior–posterior intestine junction (AI versus PI), revealing the abrupt change of epithelium (arrowhead), from simple columnar (at left) to stratified columnar (at right). Bar = 100 μ m. These images were modified from Chatchavalvanich et al. (2006). Refer to original manuscript for more details. Modified and reprinted with the generous permission of Springer-Verlag and R. Marcos.

griseus; Michelangeli et al., 1988), the Atlantic stingray (*Dasyatis sabina*; Smolka et al., 1994), and finally the freshwater whip ray (*Himantura signifier*; Chatchavalvanich et al., 2006). The reason behind this apparent species-specific evolution of gastric cells in elasmobranchs is not currently known.

Interestingly, mammalian parietal cells use secretory canaliculi and a tubulovesicular system for the secretion of HCl, while it appears that the parietal cell equivalent in the sixgill shark is lacking the secretory canaliculi system (Michelangeli et al., 1988). There was no mention as to the presence or absence of the secretory canaliculi system in the other studies on elasmobranchs with seemingly distinct gastric cells; however, tubulovesicles were observed (Smolka et al., 1994; Chatchavalvanich et al., 2006). Additionally in mammalian stomachs, chief cells and parietal cells intermingle spatially in the integument, while at least in the sixgill shark there is a definite zonal separation of the parietal cell equivalents and the chief cell equivalents (Michelangeli et al., 1988). In elasmobranch species where combined oxynticopeptic cells are found, gastric glands may also exhibit zonation, being found primarily in the cardiac and fundic regions, and not the pylorus (Grabowski et al., 1995); suggesting a gradient of proteolytic enzymes and HCl along stomach in either case (Fig. 6.4B vs. C; Chatchavalvanich et al., 2006).

3.4. The Intestine and Associated Structures

Posterior to the stomach is the intestinal portion of the GIT separated from the pyloric stomach by a circular band of muscle fiber forming the pyloric valve or sphincter. In some species there is a small chamber that the pyloric valve opens into, known as the bursa entiana (Fig. 6.3B and C). The purpose of this chamber is unclear, although in the coffin ray (*Hypnos monoterygius*) there is a partition separating the bursa entiana into two further smaller chambers (Daniel, 1934). The intestine is broken down into two sections: the proximal small intestine (or anterior intestine or duodenum), and the distal large intestine (or posterior intestine or spiral intestine; Fig. 6.3B–D). In a few species of elasmobranchs there are one to several large appendages attached to the anterior intestine (Holmgren and Nilsson, 1999) similar to pyloric caecae seen in teleost fish. The function of the caecae in sharks is unclear; however, in teleost they may increase surface area for absorption, and aid in lipid absorption (Buddington and Diamond, 1987) and possibly osmoregulation (Veillette et al., 2005). The epithelial lining of the small intestine consists of simple absorptive columnar cells with microvilli, goblet cells with neutral and acid mucins, and enterochromaffin cells (Fig. 6.4D; Chatchavalvanich et al., 2006).

The epithelium is folded (Fig. 6.4D) presumably to increase surface area of the short anterior intestine.

The anterior intestine is also the location of connections to the liver and pancreas, and hence where biliary and pancreatic secretions occur. The liver is a large, bi-lobed organ that is the location of urea synthesis and amino acid catabolism in elasmobranchs (Fig. 6.3A). These products are of utmost importance in the osmoregulatory and nitrogen balance strategy of elasmobranchs (see Chapters 4 and 5). Associated with the liver is the gall bladder, which is where bile is stored before secretion into the anterior intestine via the biliary duct (Fig. 6.3A). Bile production rates ($1\text{--}1.5\ \mu\text{l kg}^{-1}, \text{min}^{-1}$; Boyer et al., 1976) are on par with those observed in teleost fish (Grosell et al., 2000).

The pancreas is composed of two compact, discrete, and connected lobes that empty into a common duct connected to the anterior intestine (Fig. 6.3B and C). The pancreas has both exocrine and endocrine functions. The exocrine pancreas produces digestive enzymes such as lipases and colipases (Sternby et al., 1983), and occasionally chitinases typically when insects and crustaceans are a large dietary component (Fänge et al., 1979). Gastric acid additions to the anterior intestine stimulate these secretions (Babkin, 1929, 1932), although limited work has occurred since these early studies. The endocrine pancreatic cells in sharks most likely secrete insulin, glucagon, pancreatic peptide, and somatostatin, as summarized by Holmgren and Nilsson (1999).

The large intestine (Fig. 6.3C and D) is most identifiable by the presence of the spiral valve, although in some species the folds more resemble a scroll (i.e., *S. zygaena*) (Holmgren and Nilsson, 1999). The spiral valve represents a series of integument folds traversed by blood vessels. The integument of the large intestine consists of simple columnar cells with microvilli, goblet cells with neutral and acid mucins, and enterochromaffin cells as in the small intestine (Fig. 6.4E; Chatchavalvanich et al., 2006). Little work has examined the development of the spiral valve to date. Daniel (1934) suggested that the valve first appears as a ridge along the length of the posterior intestine. This ridge then rolls up into a scroll shape and if subsequent torsion occurs this scroll shape transforms into a spiral shape. The cellular mechanisms at work are unclear and deserve investigation.

The purpose of the spiral valve is likely to increase surface area for absorption and increase digesta transit time, while decreasing the overall size of the intestine (Holmgren and Nilsson, 1999). Indeed, when the length of the intestine is compared to that expected based on scaling with teleost fish (Fig. 6.5), it is obvious that the relative intestinal length is smaller than predicted based diet and species comparisons. However the calculated relative length of the intestine should be interpreted with caution as the body

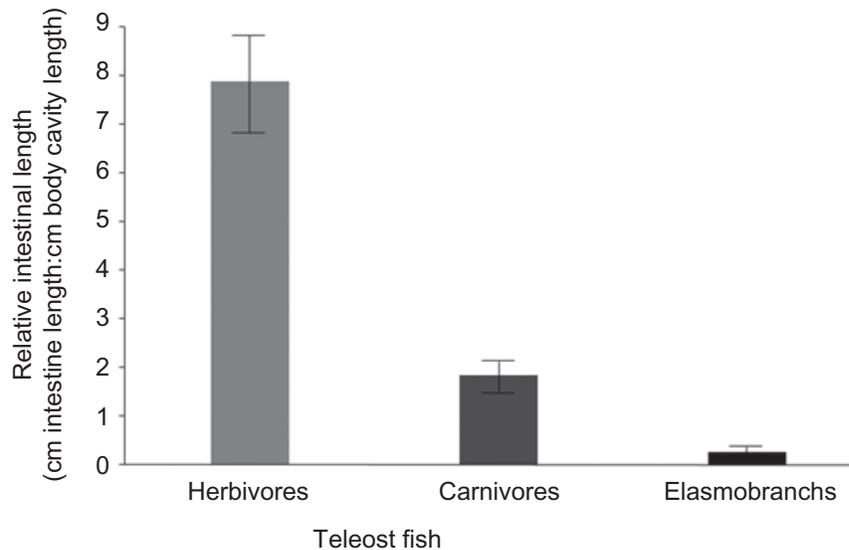


Figure 6.5. The relative length of the intestine compared to the length of the body cavity. Teleost fish are divided into herbivorous and carnivorous fish, while elasmobranchs are grouped together regardless of dietary makeup. Data from personal measurements (C. Bucking, unpublished) or recalculated from Kapoor et al. (1975) for teleost fish and from Holmgren and Nilsson (1999) for elasmobranchs.

cavity of sharks is morphologically distinct from many teleost fish, as it is fusiform to increase swimming efficiency while many teleosts have broader and/or shorter body cavities. Regardless, it is generally accepted that elasmobranch have a shorter than expected intestinal length (Holmgren and Nilsson, 1999). The spiral valve, in theory, slows down the progression of digesta through the intestine, forcing it to wind through the scrolls before elimination. This increase in processing time may allow for sufficient nutrient absorption (Sibly, 1981). One would predict that animals habitually consuming prey items of lower nutrient quality would have a slower transit time and more numbers of spirals. Supporting this is evidence that the number of turns that spiral valves form appears to be species-specific; however, there is evidence of individual variation within a species as well (Holmgren and Nilsson, 1999). The absorptive columnar cells with villi that project into the lumen of the GIT lining this section support the role of the spiral valve in absorption (Chatchavalvanich et al., 2006).

3.5. The Colon, Rectum, and Rectal Gland

The final portion of the GIT is the colon and rectum (Fig. 6.3C and B). The colon is notable for an extensive muscular layer in contrast with the intestine, as well as a transition away from columnar cells with microvilli to

stratified columnar and/or cuboidal cells (Fig. 6.4F; Holmgren and Nilsson, 1999; Chatchavalvanich et al., 2006). The epithelial goblet cells also transition toward sulfated acid mucin staining cells (Chatchavalvanich et al., 2006) and their quantity suggests they aid in passing undigested material out of the GIT. A lumen from the rectal gland demarcates the colon from the rectum. The rectal gland is a digitiform organ attached to the GIT and functions in aiding osmoregulation (Fig. 6.3D; Burger and Hess, 1960). The skate rectal gland is physically much smaller than the shark rectal gland, and the secretagogues stimulating chloride secretion differ from those seen in sharks (Kelley et al., 2014). Importantly, the rectal gland function may also be influenced by the process of digestion, with digestion triggering an increase in NaCl secretion (Wood et al., 2008, 2010), and anticipatory transcriptomic responses were recently observed in response to feeding (Deck et al., 2013).

3.6. Ontogeny of the Gastrointestinal Tract

The development of the GIT of elasmobranchs has been restricted to a single study in the southern stingray, *Dasyatis americana* (Hamlett et al., 1996). It appears that the fetal alimentary organs function early in the development of the stingray, to digest and absorb nutrient histotroph produced by the maternal uterus to aid in growth (Hamlett et al., 1985).

4. DIGESTIVE ENZYMES AND SECRETIONS

One of the roles of the GIT is to breakdown incoming food into nutrients for absorption. This is accomplished by a suite of enzymes that may be secreted in distinct portions of the GIT. For example, gastric acid secretion occurs exclusively in the stomach, while bile secretion occurs specifically in the anterior intestine. These enzymes and other secretions are essential for efficient and optimal digestion of prey, and the type of prey consumed or other aspects of feeding ecology often influence their expression.

4.1. Stomach Enzymes and Secretions

The stomach is often the site of the initiation of digestion. It allows the consumption of large quantities of food, forming a holding area where acids and enzymes can begin the process of breaking down ingested material. The stomach then meters out the digesta to the intestine for further digestion.

It is theorized that an acid-secreting stomach first appeared ~350 million years ago in the elasmobranchs (Koelz, 1992). Most vertebrates,

elasmobranchs included, secrete an acidic fluid into the stomach lumen that is primarily made of three components: gastric acid (HCl), acid proteases (pepsinogen/pepsin), and mucous. Through chemical and enzymatic catabolism this fluid aids digestion of prey residing in the stomach. One of the primary secretions associated with the stomach is gastric acid. The cell responsible for gastric acid secretion is either the oxynticopeptic cell in lower vertebrates, or the parietal (oxyntic) cell in mammals. As mentioned above, it appears that several elasmobranch species have also evolved separate acid secreting cells, although their tissue distribution in the gastric mucous appears to follow a distinct pattern compared to the distribution seen in mammals (Michelangeli et al., 1988). Regardless of whether HCl secretion occurs from a distinct cell, the enzyme responsible appears relatively conserved across vertebrates. The H^+ , K^+ -ATPase is a potassium-stimulated proton translocating adenosine triphosphatase (the HKA). Typically, the HKA is located in tubulovesicle membranes that fuse with secretory canaliculi membranes when gastric acid secretion is stimulated. This exposes the transporter to the stomach lumen where intracellular protons exchange for luminal potassium ions. Chloride channels in the apical membrane of the cells allow Cl^- to enter the lumen and form HCl for digestion. The gastric HKA orthologue in the Atlantic stingray is >80% identical to the gastric HKA in mammals (Choe et al., 2004), which indicates the evolutionary conservation of this transporter across species (Smolka et al., 1994).

It is clear, that as in other vertebrates, the ingestion of a meal and subsequent digestion triggers an increase in gastric acid secretion in elasmobranchs (Sullivan, 1905). This gastric phase of acid secretion (Olsson and Holmgren, 2011), where the ingestion of food distends and/or raises the pH of the stomach (e.g., Papastamatiou and Lowe, 2004), is further controlled through secretagogues (e.g., gastrin, histamine, and acetylcholine). Evidence of an increase in pH of the stomach and subsequent lowering back to basal values following the ingestion of a meal has been directly observed in elasmobranchs (Papastamatiou and Lowe, 2004; Wood et al., 2005, 2009). Although the mechanism behind the increase is unexplained, the hypothesized mechanism behind the decreasing pH is through increased HKA activity, as a known HKA inhibitor reduced gastric acid secretion (Wood et al., 2009). Acid secretion rates in leopard sharks are pH dependent, with rates of ~ 6 mmol/h when gastric pH was > 2.5 , and ~ 2 mmol/h when pH was 2.0–2.5 (Papastamatiou, 2007).

There appears to be two approaches for gastric acid secretion in teleost fish: a continuous basal secretion that maintains a low pH in the stomach (e.g., Bucking and Wood, 2009), and a food ingestion triggered release that only lowers the pH away from neutral upon consumption of a meal

(e.g., Nikolopoulou et al., 2011). There is evidence that several species of sharks and rays employ a continuous basal secretion approach (Papastamatiou and Lowe, 2004; Papastamatiou et al., 2007; Wood et al., 2009; Anderson et al., 2010), while Papastamatiou and Lowe (2005) confirmed that nurse shark (*Ginglymostoma cirratum*) had neutral gastric pH when not holding food in the stomach. The two patterns in HCl secretion may relate to feeding patterns as frequent teleost feeders, as well as elasmobranch species that experience unpredictable food availability (Papastamatiou and Lowe, 2004, 2005; Papastamatiou, et al., 2007), tend to maintain a low gastric pH. In contrast, less frequent, sporadic elasmobranch feeders may exhibit a neutral gastric pH between feeding events (Fig. 6.1; Papastamatiou and Lowe, 2005). Yufera et al. (2012) suggests that daily feeding patterns and frequency may control gastric acidification regulation. This results in an ability to move between categories in Fig. 6.1 and represents the adaptability of the GIT to changes in feeding frequency and demands placed on the GIT in order to optimize digestion.

The secretion of gastric acid in elasmobranchs maintains a low pH in the stomach lumen, which likely aids in the activation of the main protease pepsinogen into pepsin, the other notable component of stomach secretions. Pepsinogen secretion is via the oxynticopeptic cells in the gastric mucosa of most nonmammalian vertebrates, but as with gastric acid secretion, there is limited evidence that at least some elasmobranch may have distinct cells (Michelangeli et al., 1988). Gastric acid cleaves pepsinogen into pepsin, creating an active enzyme for protein digestion. Characterization of a pepsinogen in the Portuguese dogfish (*Centroscymnus coelolepis*) revealed a monomeric protein, ~42 kDa in size, with similar characteristics to mammalian proteins (Nguyen et al., 1998), although activity rates of the elasmobranch protein are higher at low temperatures compared to mammalian proteins (Guerard and Le Gal, 1987). Unfortunately, the sequence of pepsinogen is not known in elasmobranchs.

There is evidence of chitinases and high chitinolytic activity in the stomachs of the velvet belly lanternshark (*Etmopterus spinax*) and the thorny skate (*Raja radiata*; Fänge et al., 1976; Fänge et al., 1979), although no follow-up work has been done exploring this. In teleost fish, the presence of chitinolytic activity may aid the digestion of the shells of crustaceans (e.g., Danulat, 1986), which is also likely in elasmobranchs. High activity levels would be predicted in the stomach and intestines of elasmobranch species that prey primarily on crustaceans (Cortés, 1999), although this has not been investigated. Interestingly, initial measurements of chitinolytic activities in the fish GIT were attributed to bacteria. However, additional experiments suggested that the chitinase activities in the digestive tract of Atlantic cod, *Gadus morhua*, were primarily derived from fish tissues (Danutat, 1986).

Recently, three different chitinase genes have been identified in the Japanese flounder (*Paralichthys olivaceus*), two of which were predominantly expressed in gastric glands (Kurokawa et al., 2004). As well, Krogdahl et al. (2005) have provided evidence of chitinase in genome of the pufferfish (*Takifugu rubripes*). This indicates that teleost fish have the ability to produce endogenous chitinase for digestion and may secrete it with other products of the gastric gland during digestion. While the source of chitinase activity in elasmobranchs remains unknown, what is clear is that relative to teleost fish, gastric chitinase activity is higher (Fig. 6.6A). In fact, chitinase activity in the stomach of elasmobranchs is 100-fold higher than that seen in the stomach of teleosts (Fig. 6.6A; Fänge et al., 1976; Fänge et al., 1979; Danulat, 1986).

4.2. Intestinal Enzymes and Secretions

Once food leaves the stomach, it enters the intestine where nutrients are further broken down and absorbed with water and other factors. The prolonged digesta evacuation time compared to teleost fish may be required for complete breakdown and absorption of nutrients in the shorter than expected intestine (Fig. 6.5). With a shorter intestine, there is less time and/or surface area to breakdown and absorb ingested material; however, the presence of the spiral valve in elasmobranchs most likely serves to compensate for this by increasing surface area. Regardless, this catabolism of nutrients occurs via the secretion of digestive fluids. Lipid metabolism is particularly prominent in elasmobranchs and fat digestion depends on three main intestinal secretions: bile salts and pancreatic lipase and colipase. These secretions also are the most studied in the literature and will be the focus of this section as examples of intestinal enzymes and secretions.

Pancreatic lipase hydrolyses triacylglycerol substrates into diacylglycerol, monoacylglycerol, and free fatty acids; is synthesized in the pancreas; and is secreted into the intestine via a duct. A survey of pancreatic lipase activities in elasmobranchs [*Dasyatis pastinaca* (common stingray), *Mustelus mustelus* (common smooth-hound), *Rhinoptera marginata* (murin), and *Rhinobatos cemiculus* (guitar fish) Smichi et al., 2012] revealed high activities in the sharks compared to other marine animals (Fig. 6.6B). Indeed, pancreatic lipase activity in the pancreas of elasmobranchs is 10- to 100-fold higher than activities observed in equivalent tissues in teleost fish and invertebrates (Smichi et al., 2012). This may reflect the importance of dietary FAs to the energy metabolism of elasmobranchs, as well as increased activity to optimize digestion (Sibly, 1981). It may also reflect the dependence of teleost fish on another enzyme for lipolysis, the bile salt-activated lipase that

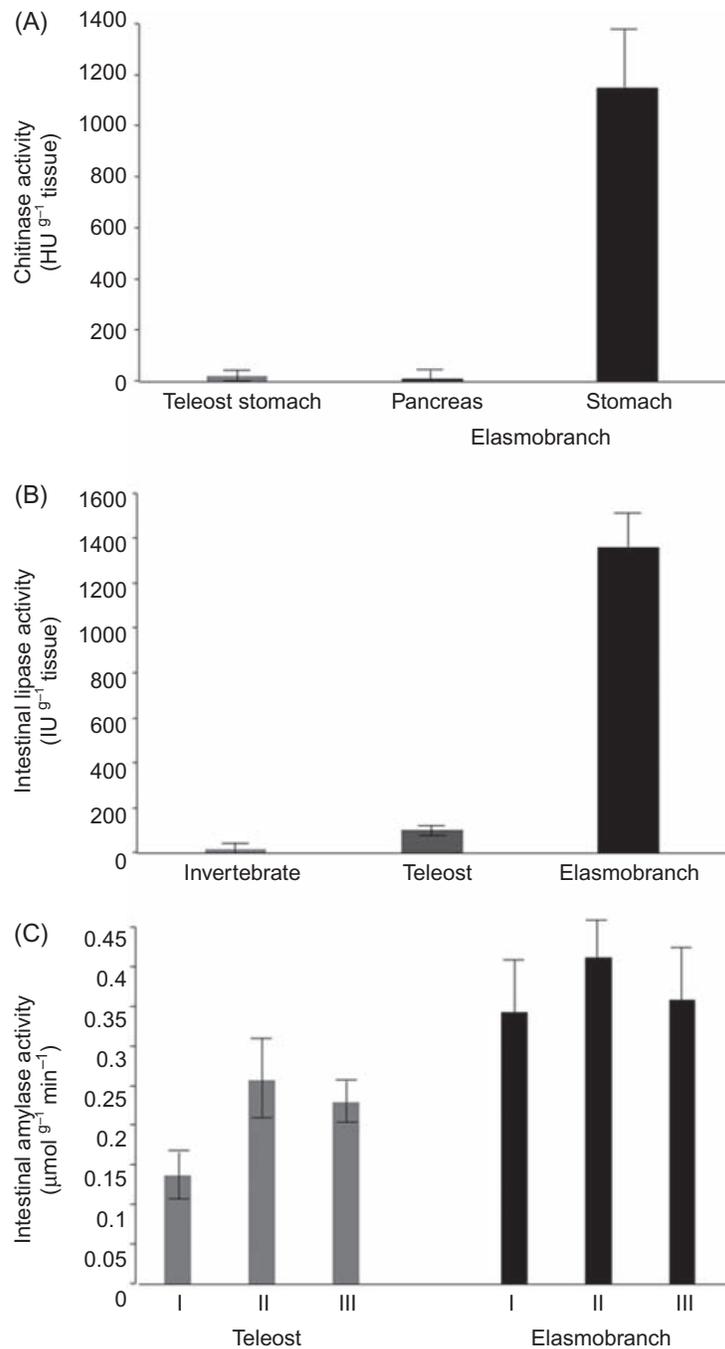


Figure 6.6. Comparative enzyme activities in the gastrointestinal tract and associated structures. (A) Chitinase activity (HU g^{-1} tissue) in the stomach tissue of teleosts and elasmobranchs and the discrete pancreas of elasmobranchs. (HU: Hulten units). Data compiled from Fänge et al. (1976, 1979). Values are means \pm S.E. (B) Lipase activity (IU g^{-1} tissue) in the pancreatic (or equivalent) tissues of invertebrates, teleost fish, and elasmobranch fish. Data compiled from Smichi et al. (2012). Values are means \pm S.E. (C) Amylase activity ($\mu\text{mol g}^{-1} \text{min}^{-1}$) in the intestinal sections of teleost fish and elasmobranchs. Intestinal sections were broken up from anterior to posterior sections into: proximal (I), middle (II), and distal (III) sections. Teleost data compiled from Chakrabarti et al. (1995), elasmobranch data compiled from Kuz'mina and Gelman (1997). Values are means \pm S.E.

hydrolyses carboxyl ester bonds of acylglycerols, cholesterol esters, and fat-soluble vitamin esters. Unfortunately, limited data exists on species-specific enzyme activity levels in elasmobranchs restricting comparisons. The common smooth-hound, the murin, and the guitar fish display similar pancreatic lipase activity levels, while the activity in the common stingray was an order of magnitude higher (Smichi et al., 2012). Future experiments on different elasmobranch species will reveal any dietary correlations within elasmobranchs and the functional significance of this observation. The pH optimum for elasmobranch lipase activity was between pH 8–9 (Smichi et al., 2012), generally in accordance with the measured pH of the intestine of elasmobranchs (e.g., Anderson et al., 2010).

Elasmobranch pancreatic tissues were positive for the co-enzyme colipase (e.g., Smichi et al., 2012), which optimizes pancreatic lipase activity (e.g., Erlanson-Albertsson, 1992), and colipase was detected in the transcriptome of the lesser spotted catshark (*Scyliorhinus canicula*; Mulley et al., 2014). Further molecular characterization of colipase from the common stingray and dogfish shark revealed a considerable (~55%) molecular identity of the enzymatically active N-terminal sequence with mammalian colipases (Sternby et al., 1984; Bacha et al., 2011). Analysis of the Atlantic cod, Japanese pufferfish, Japanese rice fish (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), and zebrafish (*Danio rerio*) genomes failed to reveal the presence of a colipase (Saele et al., 2010), which further supports the hypothesis that teleost fish appear primarily dependent on a bile salt dependent lipase (e.g., Murray et al., 2003).

Bile salts and alcohols act as detergents and serve to solubilize the lipid droplets before pancreatic lipase (aided by colipase) digests them. In mammals, bile salts are the major solutes secreted into bile, whereas in elasmobranchs and other fish, sulfated bile alcohols predominate. Hence, the alcohols are considered more primitive in comparison (Hagey et al., 2010). The major bile alcohol in elasmobranchs, scymnol sulfate, occurs at concentrations rivaling those of mammalian bile salts (Karlanganis et al., 1989). Enteral recovery of scymnol sulfate is high (>90%) in skates, although the fate of this absorbed bile salt is unknown (Fricker et al., 1997). It is possible it is recycled in the liver, or secreted via the kidney. The inhibition of cholesterol crystal formation by scymnol may be responsible for the absence of gallstones in elasmobranchs (Gilloteaux et al., 2013), although this deserves further investigation. Control of bile secretion appears to be via a gastrin- or cholecystokinin-like peptide (see Chapter 8) as in other vertebrates (Andrews and Young, 1988). However, acetylcholine did not affect gall bladder secretion of bile, despite being a potent stimulant in other vertebrates, suggesting a unique lack of neural control in elasmobranchs (Andrews and Young, 1988).

The activities of diverse nutrient catabolizing enzymes in elasmobranchs are similar to those seen in teleosts. For example, both trypsinogen activity, for protein digestion, (Zendzian and Barnard, 1967), and amylase activity, for starch and glycogen digestion, (Fig. 6.6C; Kuz'mina and Gelman, 1997) are only marginally elevated in elasmobranch GIT tissues. Enzymes involved in either carbohydrate or protein catabolism scale proportionally to dietary carbohydrates and proteins in teleost fish (Chakrabarti et al., 1995; Kuz'mina and Gelman, 1997). It remains to be seen if this is true for elasmobranchs. Ultimately, the observed increased levels of enzyme activity (Figs. 6.6A–C), together with a long passage time of digesta through the GIT, may represent adaptations to the shorter intestine found in elasmobranchs (excluding the spiral valve) in order to optimally digest their prey (Sibly, 1981). Additionally, any observed difference in GIT digestive physiology may represent trade-offs or adaptations to a variety of factors including feeding strategies (Fig. 6.1) and/or prey choice.

5. EFFECTS OF DIGESTION ON HOMEOSTASIS

Though a majority of research characterizes the underlying biochemical mechanisms, digestion exerts consequences on animal homeostasis and until recently, exploration of this phenomenon in elasmobranchs was limited. Specifically, studies examining the effect of digestion on nitrogen, ion and water, and acid/base balance in sharks have revealed interesting results.

Digestion produces ammonia through protein catabolism, both in the blood through tissues and organs and in the intestine. In ammoniotelic teleosts, digestion increases plasma ammonia levels (e.g., Bucking and Wood, 2008; Bucking et al., 2009). However, this dietary ammonia supports urea synthesis in ureotelic elasmobranchs, through the enzyme glutamine synthetase (GS). GS traps ammonia for production of urea in the ornithine-urea cycle. Interestingly, elasmobranchs exhibit a postprandial increase in plasma ammonia levels; however, it is blunted in size compared to teleosts (e.g., Wood et al., 2005; Kajimura et al., 2006 vs. Bucking and Wood, 2008; Bucking et al., 2009). After feeding, a transient increase in both urea and plasma trimethylamine oxide levels occurs (e.g., Kajimura et al., 2006; Wood et al., 2010), which has implications for osmoregulation (see Chapter 5). An increase in GS and other nitrogen metabolizing enzymes (both at the transcript and activity levels) is observed in dogfish following feeding (Kajimura et al., 2006; Walsh et al., 2006; Deck et al., 2013), revealing a potential benefit for osmoregulatory pathways in the ureotelic dogfish.

Indeed, Wood (2001) hypothesized that ammonia from digestion would be scavenged to contribute to urea production as dogfish sharks only feed sporadically and are thus nitrogen limited for urea production. Further supporting this hypothesis is evidence that rate of urea excretion does not change during digestion, indicating retention of nitrogen after feeding (Kajimura et al., 2006).

An increase in plasma urea (Kajimura et al., 2006; Wood et al., 2010) during digestion occurs in spite of a large secretion of urea into the GIT (Wood et al., 2007). Recent studies have potentially shown a complex urea-recycling program in the GIT. When fasting tissues were examined, urea was absorbed in all sections of the GIT except the intestine, where it was secreted (Liew et al., 2013). Upon feeding, there was a reversal from secretion to absorption in this section as well. This is in contrast to earlier studies on whole animal responses to feeding where a large net secretion of urea into the intestine was observed (Wood et al., 2007), possibly stemming from pancreatic and biliary secretions. The observed *in vitro* absorption then indicates an attempt by the intestine to scavenge the urea before elimination (Liew et al., 2013). Phloretin, ouabain, and sodium-free solutions inhibit urea transport in the dogfish intestine, indicating that urea is most likely dependent on several transport pathways; which supports the hypothesis that urea recycling is occurring in order to minimize nitrogen loss (Anderson et al., in press).

Little work exists examining ion and water transport pathways in the elasmobranch GIT. A recent study (Liew et al., 2013) has shown that the stomach was a site of sodium absorption following a meal, which is similar to work done in teleost fish (Bucking and Wood, 2007). In particular, there is little evidence for significant uptake of the divalent cations calcium and magnesium in any section of the GIT, whereas sodium, chloride, and water were absorbed in the intestine in both fasted and fed elasmobranchs (Wood et al., 2007; Anderson et al., 2007, 2010; Liew et al., 2013). In contrast, potassium was secreted in the intestine and was not affected by feeding (Anderson et al., 2007, 2010; Liew et al., 2013); however, this may be reflective of the *in vitro* technique itself as it contradicted earlier whole animal observations (Wood et al., 2007). Drinking, albeit at a low rate, has been suggested to occur in feeding elasmobranchs (Wood et al., 2007) possibly to help maintain osmotic balance between the GIT lumen and the plasma. The salt regulating function of the rectal gland is influenced by digestion, with an increase in NaCl secretion occurring post-meal ingestion (Wood et al., 2008, 2010). This response involves up-regulation of enzymatic activities (Walsh et al., 2006) but also transcriptomic responses (Deck et al., 2013), and presumably aids in eliminating an excess of salts absorbed during digestion.

Finally, digestion creates a systemic alkalisation of the blood or an alkaline tide, in animals that employ gastric acid digestion (Niv and Fraser, 2002). Briefly, the production of a proton that is secreted into the stomach lumen for gastric acid formation necessitates the equimolar production of a base (HCO_3^-) that is secreted into the plasma to maintain pH balance in the cell. The previously observed addition of base to the blood of elasmobranchs during digestion verifies an alkaline tide is present (e.g., Wood et al., 2005). The role of the secretion of protons into the stomach lumen has been confirmed by inhibiting the activity of HKA in the stomach using a pharmacological agent, which results in the reduction of the excess base recorded in the plasma (Wood et al., 2009). This excess base results in an increase in base excretion to the water during feeding (Wood et al., 2005, 2007), and is attenuated by the reduction of gastric acid secretion (Wood et al., 2009). The relocation of H^+ -ATPase transporters from cytoplasmic storage vesicles to the basolateral membrane of gill cells seen during digestion (Tresguerres et al., 2007; Roa et al., 2014) may drive the excretion of base to the water. Essentially, the excretion of protons into the blood may create an electrochemical gradient such that base (generated in equimolar concentrations by branchial carbonic anhydrase) is secreted to the environment.

6. FUTURE PERSPECTIVES

1. There is a need for physiological data to extend the utility of techniques such as SIA and QFASA. In particular, evidence of how elemental isotopes/fatty acids behave in biological systems is needed to avoid violating assumptions built into the analysis. Currently most evidence is from model organisms outside of Elasmobranchii, and there have been only limited investigations into chondrichthyes in particular. In order to take advantage of these techniques careful experimental studies on isotope and fatty acid tissue incorporation are needed in elasmobranchs. The confounding effects of environment (temperature, salinity, etc.); age; growth; dietary shifts; and nitrogen excretion can affect the calculated models and thus should be studied due to unique aspects of elasmobranch physiology.
2. Additional work is required regarding GIT enzyme activities in elasmobranchs. We know little about the enzyme activities in sharks, in stark contrast with the body of literature on teleost fish. Trypsin, elastase, carboxypeptidase, are just a few examples of digestive enzymes that are the potential subjects of future studies, in addition to continuing

work on lipase, colipase, and amylase discussed above. Species-specific differential expression, zonation along the GIT, and contrasts with other aquatic ectotherms are all potential areas of focus as well.

3. There is a need to know more about how the elasmobranch intestine responds to dietary shifts. Studies focusing on teleost fish show that dietary shifts are tolerated by some species with little compensation, while other species show tremendous plasticity. The response of elasmobranch intestines to dietary shifts is a fruitful area of experimental work, with little information currently existing. A large body of work on dietary prey composition shows how flexible some elasmobranch species are for the types of prey consumed. How this affects the GIT physiology may reveal how and why this variety of prey is tolerated by elasmobranchs.
4. Further work is needed to understand the interactions between whole animal and GIT physiology. For example, most elasmobranchs are ectotherms and the core temperature of these fish matches the surrounding water temperature unlike homeotherms. As ectotherms, temperature dependent processes in elasmobranchs (such as GIT enzyme activity and transport rates) will depend on the preferred water temperature that these animals inhabit, that is, the colder the water the animal is found in, the slower the reaction and transport rates. Some species of sharks generate body heat to elevate organ temperatures, and in some cases this endothermy appears to be a result of the retention of metabolic heat generated by digestion (e.g., [Carey et al., 1981, 1985](#)). In the lamnids studied so far, the temperature of the stomach (a good proxy measure for core body temperature) appears to be uniform over a small range of temperatures ($\sim 22\text{--}27^\circ\text{C}$). The gastric temperature is also elevated over, and independent from, ambient water temperatures (e.g., [Carey et al., 1981](#); [Goldman, 1997](#); [Goldman et al., 2004](#); [Béguér-Pon et al., 2012](#)). The current assumption, that this elevated temperature enhances the rate of digestion and assimilation ([Carey et al., 1981](#); [Goldman, 1997](#)), remains to be investigated thoroughly. Other aspects of whole animal physiology, such as ion or water balance, remain to be comparatively explored in elasmobranchs as well.

ACKNOWLEDGMENTS

Thanks to W.G. Anderson for providing quality pictures of the elasmobranch gastrointestinal system. Additionally, thanks to R. Marcos for permission to reprint and modify the histology figures. Finally, thanks to P. Bucking for editing and referencing help.

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METABOLISM OF ELASMOBRANCHS (JAWS II)

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The metabolism of chondrichthyan fishes is of interest because it is unlike that of any other vertebrate group with the possible exception of the coelacanth. It is dominated by the osmotic strategy of urea retention. This involves continuously synthesizing and maintaining large amounts of urea and thus impacts not only their nitrogen metabolism but also, indirectly, the metabolism of lipids and ketone bodies. The chondrichthyans may be the most nitrogen limited vertebrates. The system for synthesizing urea requires glutamine as the nitrogen donor and thus nitrogen metabolism is focused on providing this amino acid. The absence of albumin in the group precludes the rapid transport of non-esterified fatty acids for muscle contraction. Consequently, lipid-derived ketone bodies replace fatty acids as the energy source in muscle and heart. As the most primitive extant jawed vertebrate group, the chondrichthyans show advances over the jawless fish, the agnathans. They provide insight into the early stages of vertebrate evolution where food intake and digestion improved; metabolic organization started to become more specialized, with more tissue-specific protein isoforms that allowed better control of metabolism; and improved efficiency and rates of metabolic processes. Compared to higher vertebrates, however, the chondrichthyans display a simpler metabolic organization and simpler regulatory mechanisms that can help us understand the current organization of metabolism in our own species. They display fewer tissue-specific isoforms, fewer signaling molecule types, fewer hormones, and fewer multimeric enzymes compared to more advanced vertebrates. With a 400+ million year history, the group has diversified within itself, both anatomically and metabolically, in ways that are still poorly understood.

1. INTRODUCTION

My original review of the metabolism of elasmobranchs, entitled “*Jaws: the inside story. The metabolism of elasmobranch fishes*” was published in 1997 (Ballantyne, 1997). While I was compiling the existing literature on the metabolism of elasmobranchs, I came to realize just how unusual elasmobranchs are metabolically when compared to other vertebrates. It was not until 2010 that two former students again reviewed the area in a paper entitled “*The unusual energy metabolism of elasmobranch fishes*” (Speers-Roesch and Treberg, 2010). While that review did an excellent job of focusing on some of the unusual features of elasmobranch metabolism, an update of the current understanding of the overall metabolism of elasmobranchs is clearly needed. Hence this review – “*Jaws II.*” Indeed

“jaws” are very germane to this chapter. The development of a new feeding apparatus, jaws, played a major role in the ability of the chondrichthyans to access new sources of food at higher rates and that necessitated other metabolic improvements.

This review provides the basics of elasmobranch metabolism but focuses on new information or ideas since 1997 with hopes it will spur research into some of the most interesting aspects of the biology of this group. It is worth pointing out that some of the novel aspects of their metabolism relate to their position in the evolution of the vertebrates. Although they are not considered to be in the main line of vertebrate evolution leading to the more advanced fishes and tetrapods and birds, they likely represent the sole surviving representatives (with the exception of the coelacanth) of a metabolic plan from which all higher vertebrates derive their pattern.

Recently studies of freshwater and euryhaline species have revealed some interesting aspects of elasmobranch biology. Of the 1000 or more species only 5% can enter freshwater compared to about 45% of teleost species. An even smaller number can reproduce in freshwater. Indeed, there may be only one species that can reproduce in both freshwater and seawater (*Dasyatis sabinia*). The basis for this has been discussed elsewhere (Ballantyne and Fraser, 2013). Ballantyne and Fraser (2013) argued there are three groups of elasmobranchs based on their capacity to deal with osmotic challenge. The first group is marine and maintains high (400–600 mM) urea in their plasma and tissues. At the other end of the spectrum are the exclusively freshwater elasmobranchs of the family Potamotrygonidae that maintain virtually no urea in their tissues and cannot enter high salinities. Then there is a third group comprising species that live in freshwater or can move between freshwater and seawater and retain osmotically significant levels of urea in the plasma and tissues. This group seems to be unable to eliminate urea from their tissues even in freshwater where the osmotic and energetic considerations would seem to demand its elimination. Thus on one hand the Potamotrygonids have eliminated urea as an osmotic component but have lost the ability to enter seawater and on the other there is a group that cannot eliminate urea in freshwater. To understand this and other aspects of the biology of the group one must delve into the details of their metabolic organization and that is the focus of this chapter. Recent data on the sister group of the elasmobranchs, the holocephalans, indicates they resemble the elasmobranchs metabolically (Speers-Roesch et al., 2006b) so for the purposes of this review the sister group of the elasmobranchs, the holocephalans, will be included where information exists and they will be collectively referred to as the chondrichthyans. Where data refers only to one group or the other, they will be dealt with separately.

2. EVOLUTIONARY CONTEXT

Chondrichthyans are the oldest extant group of jawed fishes. As such they are the first vertebrate group that we know of to do many novel things with metabolism. Molecular evidence suggests that the chondrichthyans are a terminal group that is not ancestral to the other jawed vertebrates (Rasmussen and Arnason, 1999). In spite of this, it is likely that the common ancestor had many features in common with the chondrichthyans. Thus, the sequencing of the chimaera, the “elephant shark” (*Callorhinchus milii*), genome has provided insights into the evolutionary origins of many vertebrate features (Venkatesh et al., 2014) and the pending sequence of the little skate, *Leucoraja erinacea*, will add significantly to the understanding of the metabolic organization of the group. As an ancient group, retaining many primitive features, it is not surprising that the rates of molecular evolution of elasmobranch nuclear and mitochondrial genes are an order of magnitude slower than that of mammals (Martin, 1999) and the genome of *C. milii* is the slowest evolving of all known vertebrate genomes (Venkatesh et al., 2014). See Janvier and Pradel (2015; Chapter 1, Vol. 34A) for a complete review of the systematic and evolution of the group.

The raw material for increasing metabolic complexity arises from genome duplications. Elasmobranchs have undergone only one genome duplication event subsequent to the appearance the jawless fishes because they arose before the additional genome duplications of the more advanced fishes and tetrapods. They therefore have fewer duplicate genes for developing protein isoforms, which when differentially expressed in various tissues, allow for a greater level of control and improved performance. Subcellular isoforms, it seems, were largely developed much earlier in the evolution of animal life and the chondrichthyans resemble most animals in this regard.

As representative of such an early stage in vertebrate evolution, the chondrichthyans were the first known group to develop many characteristics. These are outlined in Table 7.1 and will be elaborated on in subsequent sections. Some of these do not fall within the domain of metabolism but are worth mentioning because they indirectly involve metabolic processes. For example, they are the oldest vertebrates to use myelin to wrap their nerves and this must require the pathways for myelin synthesis. They are also the oldest known vertebrate group to have an adaptive immune system using antibodies. They have an unusual blood-brain barrier that differs from other vertebrates in that the tight junctions are between glial cells not the endothelial cells thus the glial cells have to perform functions of endothelial cells in regulating influx of metabolites.

Table 7.1
Examples of unusual or novel features related to chondrichthyan metabolism

| Feature | Consequences |
|---|--|
| First known vertebrate to have jaws | Improved feeding |
| First known vertebrate to have stomach acid secretion | Improved digestion of proteins |
| First known vertebrate to have pepsin, carboxypeptidase B, and elastase | Improved digestion of proteins |
| First known vertebrate to have an adaptive immune system | Improved disease resistance |
| First known vertebrate to have myelinated nerves | Improved speed of nerve conduction |
| First known vertebrate to have heteromeric hemoglobins with enhance cooperativity | Improved oxygen uptake and delivery |
| Lack of types I and III ITP receptors in gall bladder | Slower bile secretion and lipid digestion |
| Blood brain barrier at glial cells | Transport functions in glial cells not endothelial cells |
| Cytochrome oxidase is monomeric | unknown |
| Absence of calcium binding phosphoproteins | Inability to make bone |
| Lack of fat oxidation in muscle and heart | Ketone body oxidation is constitutive in those tissues |
| CPSase III | Glutamine required for urea synthesis |
| Simple eicosanoid signaling pathways | Poorer regulation of multiple processes |
| Fewer tissue specific isoforms | More generalized tissue specific metabolic regulation |
| Stress hormone cortisol absent – replaced by 1 α -hydroxycorticosterone | unknown |

See text for discussion and references.

The lack of calcium binding phosphoproteins in cartilaginous fish has been cited as the basis for the lack of bone in this group (Venkatesh et al., 2014) and this has consequences for the role of calcium in metabolic regulation. With a 400+ million year history some metabolic diversity has arisen within the group that complicates understanding the relationship of the metabolic organization of the group to that of other vertebrates. In spite of this, most metabolic traits are common to the entire group.

3. DIET AND DIGESTION

The need for a high protein intake may preclude the capacity to use lower protein content foods. So while 5% of the families of teleost fish are

herbivorous (Bone and Moore, 2008; Choat and Clements, 1998) no elasmobranch species are. Processing time for food is slower in elasmobranchs than in teleosts (Wetherbee and Gruber, 1990; Wetherbee and Cortes, 2004). This may impact the rate of food consumption, which has been estimated at up to 5.5% body weight per day (Wetherbee and Cortes, 2004), and is lower than the maximal rates for teleost fish (Cortes et al., 2008). The bases for this are not well established but some possible factors are explored below and in Chapter 6. For more details of the mechanics and anatomy of digestion see Cortes et al. (2008).

Digestion begins in the stomach, which is acidic, and continues to the spiral intestine, which is alkaline as in other vertebrates (Menon and Kewalramani, 1959). The chondrichthyans are the oldest known group to have the capacity to acidify then neutralize gut contents. The development of this trait accompanied by the divergence of the two genes for the hormones responsible, gastrin and cholecystokinin (CCK) (Johnsen et al., 1997). There is an increase in acid secretion in the stomach after feeding (Papastamatiou and Lowe, 2005; Papastamatiou and Lowe, 2004) but acid secretion may be continuous in some species or cease during fasting (Cortes et al., 2008). In most elasmobranch species there is a single cell type for secreting acid and zymogens (Barrington, 1942; Rebolledo and Vial, 1979) but at least one species has both parietal (acid secreting) and chief cells (zymogen secreting) (Michelangeli et al., 1988). Acidification is accomplished via a H^+K^+ ATPase (Smolka et al., 1994). There is also a protective carboxylated mucopolysaccharide protective mucous lining the stomach as in higher vertebrates (Galindez, 1992). Interestingly, the chimaera, *Callorhincus milii*, lacks a stomach and the genes for the H^+K^+ -ATPase and pepsinogen although this is thought to be a secondary loss (Castro et al., 2013). Since no older lineages (i.e., the Agnatha) also lack stomachs and the genes for the proton pump and pepsinogen, it is possible the elasmobranchs evolved the stomach and associated physiology after divergence from the chimaeras.

It has been observed that elasmobranchs can generate lower pHs in the stomach than teleosts (Lobel, 1981; Montgomery and Pollak, 1988; Papastamatiou and Lowe, 2004; Kidder, 1991). Most vertebrates can only secrete HCl isotonic to their body fluids (Johnson, 1985) but due to the higher osmolarity of their body fluids elasmobranchs can secrete more HCl and thus generate a lower pH in the stomach (Cortes et al., 2008). Furthermore, acid secretion in the elasmobranch stomach is not reliant on a membrane potential across the acid secreting cells as it is in other vertebrates (Hogben, 1959).

Somatostatin occurs in elasmobranch intestinal cells and may play a role in decreasing acid secretion if intestinal pH is too low (Cortes et al.,

2008; Conlon et al., 1985). Substance P induces contractions of the stomach *in vitro* (Andrews and Young, 1988; Holmgren, 1985; see Chapter 8) for more detail on the hormonal control of digestion.

The spiral valve intestine is shared with coelacanth, bichirs, lungfish, bowfins, sturgeons, paddlefish, gars, and the primitive teleosts, the osteoglossids. Its absorption efficiency is comparable to that of teleost fishes (Cortes et al., 2008). The chyme coming from the stomach is neutralized by the secretion of bicarbonate by the pancreas (Cortes et al., 2008) under the control of CCK. Food can be retained in the spiral valve by retrograde contractions. The role of the rectum may be mainly for reabsorption of nitrogen because levels of urea are lowest there compared to other parts of the gut (Anderson et al., 2010). Based on higher levels of expression of mRNA for the Rhesus like ammonia transporter compared to that of a urea transporter, ureolytic bacteria may play a role in converting urea to ammonia, which is then taken up (Anderson et al., 2010). See Chapter 5 for more detail regarding urea dynamics, the transport functions, and acid–base physiology of the gut.

3.1. Carbohydrate Digestion

The reliance on, or need for, carbohydrate can be assumed to be low in chondrichthyans due to the carnivorous diet and the reliance on lipid-derived ketone bodies as a fuel for many of the tissues that use carbohydrates in other vertebrates. Amylase and saccharase have been detected in intestinal mucosa of elasmobranchs at similar levels to those of teleost fish (Kuz'mina, 1990). Fourteen glucosidases have been reported from the intestinal mucosa, pancreas, spleen, and epigonal organs of elasmobranchs (Fange et al., 1980). The role of glucosidases in the epigonal organ may be for digestion of bacteria as part of the immune defense system (Fange et al., 1980). Nothing is known of sugar transport in the gastrointestinal system of chondrichthyans.

3.2. Protein Digestion

Although teleost fish are more 2–20 fold more efficient at assimilating dietary protein than birds or mammals (Smith et al., 1978) it is not known if the same applies to chondrichthyans. Certainly, their need for nitrogen would suggest they would benefit from being more efficient converters of protein but this has not been established. Some of the features responsible for the efficiency of teleost fish digestion should apply to elasmobranchs. These include the reduced cost of a lower mass-specific metabolic rate. A higher efficiency of conversion of dietary protein in teleosts is due to a

slower turnover of existing protein (Huisman, 1976). This may apply to chondrichthyans but it is not known if this is the case. On the other hand, while the reduced cost of excreting nitrogen as ammonia helps make teleosts more efficient, the high cost of urea synthesis in elasmobranchs would reduce their efficiency.

Chondrichthyans are the first known vertebrates to use pepsin, carboxypeptidase B, and elastase for protein digestion (Nilsson and Fange, 1970; Fange and Sundell, 1969). This innovation, along with an acid stomach, may have improved the rate of protein digestion and with jaws to provide a more efficient way of taking in food, and this may have contributed considerably to the success of the group. The protein digestive enzymes of elasmobranchs are similar to those of other higher vertebrates. Pepsinogen is produced in the stomach and is cleaved to pepsin at pH 4.0 (Ash, 1985). Four pepsinogens have been identified in *Mustelus canis* (Merrett et al., 1969). Carboxypeptidase A (Lacko and Neurath, 1970) and B (Prah and Neurath, 1966b) have been characterized from the pancreas of *S. acanthias* and carboxypeptidase B from *Scyliorhinus canicula* (Hajjou et al., 1995). Chymotrypsinogen and trypsinogen are produced in the pancreas as in other vertebrates and secreted into the intestine where they are activated by the alkaline pH. Chymotrypsinogen (Prah and Neurath, 1966a) and chymotrypsin have been characterized from the pancreas of *S. acanthias* (Prah and Neurath, 1966a; Ramakrishna et al., 1987).

3.3. Chitin Digestion

Chitin can represent 20% of the available energy in crustacean prey (Clements and Raubenheimer, 2006). Those elasmobranchs that feed on crustaceans also benefit from the capacity to access the nitrogen in the chitin. Chitinase is found in the stomach mucosa of many elasmobranchs (Fange et al., 1979; Micha et al., 1973). The chitinase of *S. acanthias* has an acid pH optimum as would be expected based on its site of action (Fange et al., 1979).

3.4. Lipid Digestion

Lipids are an important component of the diet for chondrichthyans because they can be used as an energy source to spare nitrogen and are needed for buoyancy control (see section 7.1). Lipid digestion requires an emulsifying agent to solubilize lipids as well as an enzyme to begin the breakdown of ingested lipids. The main emulsifying agent is the bile alcohol 5 β -scymnol 27-sulfate (3 α ,7 α ,12 α , 24,26,27-hexahydroxy-5 β -cholestan-27-sulfate) in elasmobranchs and 5 β -chimaerol (3 α ,7 α ,12 α ,

24,27-pentahydroxy-5 β -cholestane) in chimaeras (Hagey et al., 2010; Hofmann et al., 2010; Moschetta et al., 2005). These compounds are unique to the Chondrichthyes (Pettigrew et al., 1998a). A sulfotransferase involved in adding the sulfate to 5 β -scymnol and has been characterized from the cytosol of the liver (Pettigrew et al., 1998a) and kidney and testis (Pettigrew et al., 1998b) of *Heterodontus portusjacksoni*.

Concentrations of scymol sulfate in the bile of *L. erinacea* rivals that of mammals (25 mM) (Karlaganis et al., 1989). Bile production is continuous but slow in elasmobranchs (Boyer et al., 1976). The slow rate of bile secretion in elasmobranchs has been attributed to a slower, more primitive, calcium signaling system in the hepatocytes (Nathanson et al., 1999). The basis for this appears to be the expression of only one inositol 1,4,5 triphosphate receptor (Type I) instead of the three found in mammalian liver cells. The receptor is distributed throughout the cell membrane and not as highly polarized as in mammals where it is largely apical (Nathanson et al., 1999). The lack of Type II and III receptors may further reduce the responsiveness of the cells because these receptors act as feed-forward activators of calcium influx and signaling events (Nathanson et al., 1999).

The digestive lipases of elasmobranchs have a positional specificity for the 1 and 3 positions (Brockerhoff and Hoyle, 1965; Brockerhoff et al., 1968) as in most animals. The lipase from *Triakis semifasciata* has a requirement for a bile salt (Patton et al., 1977). A colipase is required to protect the lipase from the bile salts and is present in chondrichthyans as in all other vertebrates (Sternby et al., 1983). A colipase has been characterized from *S. acanthias* and has properties similar to that of other animals (Sternby et al., 1984). A pancreatic nonspecific lipase that may be involved in wax ester hydrolysis has been identified in *Triakis semifasciata* (Patton, 1975).

3.5. Specific Dynamic Action

The magnitude of the specific dynamic action (SDA) is 2-3 fold higher than resting rates in *Scyliorhinus canicula* (Sims and Davies, 1994) and 2.2 fold higher in *Chiloscyllium plagiosum* (Chen et al., 2008). This is similar to the range reported for teleost fish (1.5–2.5) (Jobling, 1981). SDA is 6–12.5% of consumed energy in *Scyliorhinus canicula* (Sims and Davies, 1994) and 6.3–12.8% in *Chiloscyllium plagiosum* (Chen et al., 2008). This is lower than that of teleosts (12–16%) (Brett and Groves, 1979), which implies a more efficient digestive process in elasmobranchs.

4. OXIDATIVE METABOLISM

The use of oxygen by chondrichthyans has been examined from the whole animal down to the level of the protein cytochrome oxidase (CCO). The efficiency of the use of oxygen at each level has not been extensively examined. One study has shown the efficiency of coupling ion transport to oxidative metabolism in elasmobranchs is higher than that of other organisms. Based on the number of ions transported per oxygen consumed, the dogfish rectal gland is the most efficient of 11 ion-transporting epithelia (Mandel and Balaban, 1981). The effects of environmental factors such as salinity or temperature on oxidative metabolism have hardly been examined. One of the very few studies of the effects of temperature acclimation on chondrichthyans indicates at least in tropical elasmobranchs there is a limited capacity at the whole animal and enzyme level to adapt to changing environmental temperature (Tullis and Baillie, 2005).

4.1. Respiration Rates

Although early studies suggested the metabolic rates of elasmobranchs are lower than those of teleosts (Brett and Blackburn, 1978) other studies indicate the metabolic rates of non-endothemic sharks are more comparable to those of teleosts (Bushnell et al., 1989). The methods used and the standard metabolic rates of a variety of elasmobranchs ranging from more sluggish benthic skates to the highly active endothemic sharks have been summarized elsewhere (Carlson et al., 2004). These indicate a tenfold range in standard metabolic rates within the group (Carlson et al., 2004). The standard metabolic rate for endothemic mako sharks is comparable to that of ectothermic sharks although their maximal metabolic rates are the highest of any shark species (Sepulveda et al., 2007). Diurnal rhythms in respiration rate occur in elasmobranchs (Sims et al., 1993). See Chapters 1, 2 and 3 for more information on circulatory systems, respiration, and gas transport respectively.

4.2. Oxygen Delivery and Hypoxia

Oxygen delivery to the tissues is a key component in determining how fast aerobic processes can occur. The chondrichthyans display some advances that allow faster rates of respiration over those of agnathans. The extant agnathans (hagfish and lampreys) only have monomeric oxygenated hemoglobins that can form homopolymeric dimers or tetramers with weak cooperativity upon deoxygenation (Qiu et al.,

2000). Heteropolymers of hemoglobins with α and β subunits and strong cooperativity arose either in the chondrichthyans or in their jawed ancestor (Gribaldo et al., 2003). This innovation with its associated enhanced cooperativity improves the efficiency of loading and unloading hemoglobins, which is needed in a more active predator. Faster loading and unloading allows faster and more efficient blood circulation for delivery of metabolites for oxidation and ATP synthesis. The regulation of hemoglobin oxygen binding and carrying capacity is not different from that of teleost fish except for the absence of a Root effect (Wells and Weber, 1983). Because chondrichthyans lack an oxygen-filled swim bladder there is no need for a Root effect.

Elasmobranchs use a variety of organic phosphates including ATP (Aschauer et al., 1985), GTP (Wells and Weber, 1983) 2,3-diphosphoglycerate (2,3-DPG) (Wells and Weber, 1983), and inositol hexaphosphate (Wells and Weber, 1983) to modulate oxygen-carrying capacity and although other organic phosphates are present they do not affect hemoglobin oxygen binding. The basis for this diversity has not been explained and may have a phylogenetic basis. See Chapter 3 for more details on the role of organic phosphates in elasmobranch erythrocytes.

A survey of elasmobranchs and teleosts found the hematocrit of elasmobranchs is lower than that of teleosts (41.0%) with skates having lower values than sharks (17.9% vs 24.8%) (Filho et al., 1992). Elasmobranch erythrocytes are about 3-fold larger than those of teleosts (Filho et al., 1992). Thus while the hematocrit values of elasmobranchs are about half those of teleosts, the hemoglobin levels are only about 70% those of teleosts (Filho et al., 1992). During exercise there is only a modest increase (10–20%) in hematocrit (Bushnell et al., 1982; Piiper et al., 1977; Brill et al., 2008) compared to 50–100% in exercised teleosts (Wells and Weber, 1990; Gallagher and Farrell, 1998; Yamamoto et al., 1980; Yamamoto and Itazawa, 2014). In teleosts, this capacity is due to an adrenergic splenic contraction, which is lacking in elasmobranchs (Opdyke and Opdyke, 1971). A lack of change in hematocrit in hypoxia is typical of elasmobranchs (Dabruzzi and Bennett, 2014).

Some adaptation to hypoxia is possible. In response to chronic hypoxia, *Dasyatis sabina* almost doubled its gill surface area but did not increase hematocrit or hemoglobin levels (Dabruzzi and Bennett, 2014). Ram ventilating shark species increase swimming speed in response to hypoxia while other species that use buccal ventilation reduce swimming speed (Carlson and Parson, 2001; Parsons and Carlson, 1998). The P_{crit} (critical oxygen level at which there is a switch to anaerobic metabolism) is lower for hypoxia-tolerant species such as the intertidal epaulette shark (*Hemiscyllium ocellatum*) compared hypoxia-intolerant species such as the shovelnose ray

Aptychotrema rostrata (Speers-Roesch et al., 2012b). The heart of the hypoxia-tolerant epaulette shark is able to maintain ATP and creatine phosphate levels better than that of the shovelnose ray (Speers-Roesch et al., 2012a). The hypoxia tolerant reef shark, *Hemiscyllium ocellatum* lowers its metabolic rate (including bradycardia) in hypoxia and undergoes a metabolic depression if hypoxia persists (Nilsson and Renshaw, 2005). See Chapter 3 for more detail on gas transport and exchange.

4.3. Mitochondrial Metabolism

Isolated mitochondria from elasmobranchs have been used to identify the preferred substrates of different tissues and this has been summarized in (Ballantyne, 1997). Generally, liver mitochondria utilize glutamate and acyl carnitines while red muscle mitochondria prefer glutamine and β -hydroxybutyrate (BHB) (Ballantyne, 1997). Liver mitochondria oxidize shorter chain fatty acids (6–14 carbons) better than palmitate (16 carbons) (Moyes et al., 1986b). The capacity of liver mitochondria to oxidize citrate and isocitrate is high (Moyes et al., 1986b) and similar to that of rainbow trout (Suarez and Hochachka, 1981). This indicates the capacity to transport citrate to the cytosol for lipid synthesis (see Section 7.2 for more detail). The only study of elasmobranch heart mitochondria indicates they oxidize BHB and pyruvate at high rates (Moyes et al., 1990) and although glutamine oxidation was not examined, it is likely also oxidized at high rates based on high levels of glutaminase (Chamberlin and Ballantyne, 1992). Elasmobranch red muscle and heart mitochondria are unlike those of any other vertebrate in their incapacity for lipid oxidation.

The electron transport chain of elasmobranchs retains some apparently primitive features. The cytochrome oxidase (CCO) of elasmobranchs is a monomer compared to the dimeric enzyme in mammals (Holm et al., 1996; Wilson et al., 1980; Georgevich et al., 1983; Blenkinsop et al., 1996). Thus while the monomeric form of the mammalian enzyme is inactive (Georgevich et al., 1983) the shark monomer functions similarly to the active dimeric mammalian form (Bickar et al., 1985) and the turnover number is similar (Holm et al., 1996). Postprandial increases in respiration associated with SDA include increases in enzyme activities and mRNA for CCO increases after feeding in rectal gland (Deck et al., 2013) but likely occurs in other tissues as well.

The mitochondria specific phospholipid cardiolipin is required for the function of CCO in mammals (Fry and Green, 1980). In *S. acanthias*, cardiolipin is not required and absent from purified cytochrome oxidase compared to 4–5 moles of cardiolipin per mole of CCO haem found in the bovine enzyme (Al-Tal et al., 1983). The shark is similar in this regard

to other lower organisms such as yeast (Al-Tal et al., 1983). Although the levels of cardiolipin in liver mitochondria are higher compared to agnathans, and similar to levels of teleosts (Glemet and Ballantyne, 1996), the fatty acids of elasmobranch liver cardiolipin are more highly saturated than those of any other vertebrate (Glemet and Ballantyne, 1996). Nothing is known of the metabolism of cardiolipin in chondrichthyans.

Reactive oxygen species (ROS) are likely produced at complexes I and II in elasmobranch heart mitochondria (Hickey et al., 2012) as in mammals but this can be reduced in some species. Heart mitochondria from the hypoxia tolerant *Hemiscyllium ocellatum* produce less reactive oxygen species during hypoxia compared to a hypoxia-sensitive species *Aptyotrema rostrata* (Hickey et al., 2012).

The capacity of the lamnid sharks to maintain warm red muscles has raised the question of how the heat is generated. Likely, it is the heat of oxidation of substrates for muscle contraction plus the heat of ATP hydrolysis. The possibility that the mitochondria of the warm red muscle of endothermic sharks were more uncoupled than those of non-endothermic sharks was dismissed based on state 4 rates of oxidation (Ballantyne et al., 1992). This has been confirmed by more detailed studies of the proton leak rates (Duong et al., 2006).

5. CARBOHYDRATE METABOLISM

Based on tissue hexokinase activities, red muscle, heart, brain, kidney, and rectal gland have a high capacity for glucose utilization (Moon and Mommsen, 1987; Crabtree and Newsholme, 1972).

In the brain, due to the localization of the blood brain barrier at the glial cells rather than the endothelial cells as in higher vertebrates, glial cells must control the movement of metabolites into and out of the brain. Glucose transporters such as GLUT1 have been identified in glial cells of the blood brain barrier of *Schroederichthyes chilensis* and *Scyliorhinus canicula* (Balmaceda-Aguilera et al., 2012). It is not known how this impacts neural integrity.

Chondrichthyans seem to be at an early stage in the evolution of glucose regulation. Plasma glucose levels range widely (Ballantyne, 2014) with values as high as 18 mM reported for stressed (angling stress) animals (Hoffmayer and Parson, 2001). Glucose levels fall after feeding but slowly rise to control levels (Wood et al., 2010). Glucose levels remain relatively constant during prolonged fasting (54 days for *S. acanthias*) (Wood et al., 2010) likely

due to the minimal need to use glucose. The basis for this poor regulation of plasma glucose levels is likely due, in part, to poor renal regulation. The lack of a transport maximum for D-glucose in the kidney of *S. acanthias* (Boylan and Antkowiak, 1966) would compromise the ability to reduce high glucose levels on a single pass through the kidney and result in a loss of glucose in the urine. Interestingly, the skate, *L. erinacea*, does have a transport maximum in the kidney (Althoff et al., 2007) and may be better able to regulate blood glucose. Perhaps this is due to a greater intake of dietary carbohydrate in the skate.

In mammals, two sodium glucose linked transporters (SGLT) are involved in reabsorption of glucose: a low affinity, high capacity transporter (SGLT2) expressed in proximal parts of the tubule and a high affinity transporter (SGLT1) in distal parts. This allows reabsorption of tubular glucose with a high efficiency and thus permits a more tightly regulated blood glucose. Both SGLT1 and SGLT2 have been identified in kidneys of elasmobranchs but not together in the same species (Kipp et al., 1997). A low affinity (1.9 mM) high capacity sodium-linked glucose transporter, similar to mammalian SGLT2, has been identified in *S. acanthias* kidney where it is distributed over a considerable length of the distal parts of the tubule including the collecting duct (Althoff et al., 2006). This arrangement would not be able to draw down glucose in the filtrate to very low levels. The high affinity SGLT1, but not SGLT2, has been demonstrated in the kidney of *L. erinacea* with a stoichiometry of 2 sodiums per glucose (Althoff et al., 2006). This arrangement would be able to take up glucose at very low levels. Thus elasmobranchs have evolved at least 2 different patterns of reabsorbing tubular glucose with neither being as efficient as the mammalian system.

5.1. Glycolysis

Although dietary carbohydrates may not be significant energetically, they can be important in the metabolism of many tissues. Glycogen levels in the muscle are similar to those of agnathans and teleosts while liver levels are higher than those of some teleosts (Leibson and Plisetskaya, 1968; Idler et al., 1969). Glycogenolysis is simpler than that of the higher vertebrates but more complex than that of the agnathans. The elasmobranchs are the earliest known vertebrates to have two isozymes of glycogen phosphorylase: a liver and muscle form (Yonezawa and Hori, 1979). Both are activated by AMP and the muscle form has a higher affinity for glycogen (Yonezawa and Hori, 1979). The enzyme is activated by a phosphorylase kinase with three subunits, as in mammals (Fischer et al., 1978). Elasmobranchs have a simpler mechanism of regulating glycogen phosphorylase because the

phosphorylase kinase is not subject to phosphorylation/dephosphorylation by protein kinases or phosphatases (Fischer et al., 1978). Glycogen phosphorylase is activated by calcium but not by circulating catecholamines (Fischer et al., 1978) as it is in higher vertebrates.

Some of the enzymes of glycolysis have diversified into tissue-specific forms [phosphoglucose isomerase (Al-Hassan, 1985)] but not others [glyceraldehyde 3-phosphate dehydrogenase (Fisher et al., 1980)]. The regulatory enzymes pyruvate kinase (PK) and phosphofructokinase (PFK) are found in lower activity in heart and red muscle than in teleost fish but PK is stimulated by fructose 1,6 bisphosphate (Zammit et al., 1978) and PFK is inhibited by citrate as in other vertebrates (Newsholme et al., 1977). Unlike the situation in teleosts, pyruvate dehydrogenase is not activated during exercise or recovery (Richards et al., 2003).

While agnathans have only a single lactate dehydrogenase (LDH) (Baldwin and Lake, 1987) chondrichthyans have two forms, A and B, but the C form found in higher vertebrates is absent (Coppes et al., 1990; Fisher et al., 1980). Tissues with the highest LDH activities are red and white muscle, heart, and rectal gland (Moon and Mommsen, 1987). It has been suggested that anaerobic pathways support salt secretion by the rectal gland during peak periods of activity (Walsh et al., 2006b). Postprandial salt secretion in the rectal gland may require anaerobic support and upregulation of LDH occurs (Deck et al., 2013). During exercise plasma lactate levels increase about tenfold over those of resting sandbar sharks (Brill et al., 2008), dusky sharks (Cliff and Thurman, 1984), spiny dogfish (Richards et al., 2003), and the giant shovelnose ray *Rhinobatos typus* (Lowe et al., 1995). In hypoxia-tolerant species lactate levels do not rise substantially during hypoxia (Wise et al., 1998). As indicated above the pathways for utilization of lactate is not well understood. The brain may be able to use lactate because a monocarboxylate transporter (MCT) identified as MCT2, a high affinity carrier, involved in lactate uptake into cells in mammals, has been identified in brain of *S. acanthias* (Balmaceda-Aguilera et al., 2012). MCT4 is a low affinity carrier responsible for lactate efflux from cells and has been shown to increase with hypoxia in the brain stem of sharks (Balmaceda-Aguilera et al., 2012).

5.2. Gluconeogenesis

The liver is the most important site of gluconeogenesis in elasmobranchs (Mommsen and Moon, 1987). The lack of phosphoenolpyruvate carboxykinase (PEPCK) in any tissue other than liver limits gluconeogenesis elsewhere. It has been suggested that the reliance on ketone bodies by many

tissues reduces the need for gluconeogenesis (Ballantyne, 1997). The fact that the kidney lacks PEPCK, and thus is not a significant contributor to gluconeogenesis, distinguishes the chondrichthyans from all the higher vertebrates (Moon and Mommsen, 1987).

There are a variety of possible gluconeogenic substrates. Liver cells can make glucose from alanine, glycerol, serine, and lactate with rates from glycerol being the highest (Mommsen and Moon, 1987). Amino acids may be more important than lactate as gluconeogenic substrates (Mommsen and Moon, 1987). During starvation alanine synthesis increases in muscle (Leech et al., 1979), which provides a potential gluconeogenic precursor. Gluconeogenesis from serine requires serine pyruvate transaminase, glycerate dehydrogenase, and glycerate kinase, none of which have been reported in chondrichthyans. Presumably the hydrolysis of triacylglycerols to fatty acids for the production of ketone bodies frees glycerol for gluconeogenesis. Glycerol kinase is absent from muscle, which precludes the use of glycerol for gluconeogenesis in that tissue (Newsholme and Taylor, 1969). The lack of endogenous lipid oxidation in muscle means glycerol production from triacylglycerol hydrolysis would be minimal.

The known pathways involved in hepatic gluconeogenesis from lactate, glycerol, and alanine are illustrated in Fig. 7.1. Gluconeogenesis from lactate involves cytosolic lactate dehydrogenase to convert lactate to pyruvate in the cytosol. Pyruvate is transported into the mitochondrial matrix by a monocarboxylate carrier that has not been characterized in elasmobranchs. The pyruvate is then converted to oxaloacetate by a mitochondrial pyruvate carboxylase (PC). The oxaloacetate is converted to phosphoenolpyruvate (PEP) by mitochondrial PEPCK. PEP exits the mitochondria on a carrier, perhaps the same monocarboxylate carrier used for transport of pyruvate into the mitochondria. PEP then is ultimately converted to glycogen by the reversal of the glycolytic pathway using fructose biphosphatase (FBPase) to bypass phosphofructokinase (PFK).

Gluconeogenesis from glycerol in liver (Fig. 7.1) involves using glycerol derived from the lipolysis of triacylglycerols. Glycerol is converted to glyceraldehyde-3-phosphate (G3P) by glycerol kinase. G3P is then converted to glycogen by the reversal of glycolysis again using FBPase to bypass PFK.

Gluconeogenesis from alanine in liver involves transamination of alanine via glutamate pyruvate transaminase (GPT) to pyruvate, which can then enter the same sequence as described above for gluconeogenesis from lactate. The glutamate produced by GPT enters the mitochondria on an uncharacterized carrier and is converted to α -ketoglutarate (α KG), which exits the mitochondria on an unknown carrier to regenerate the α KG used in the GPT reaction.

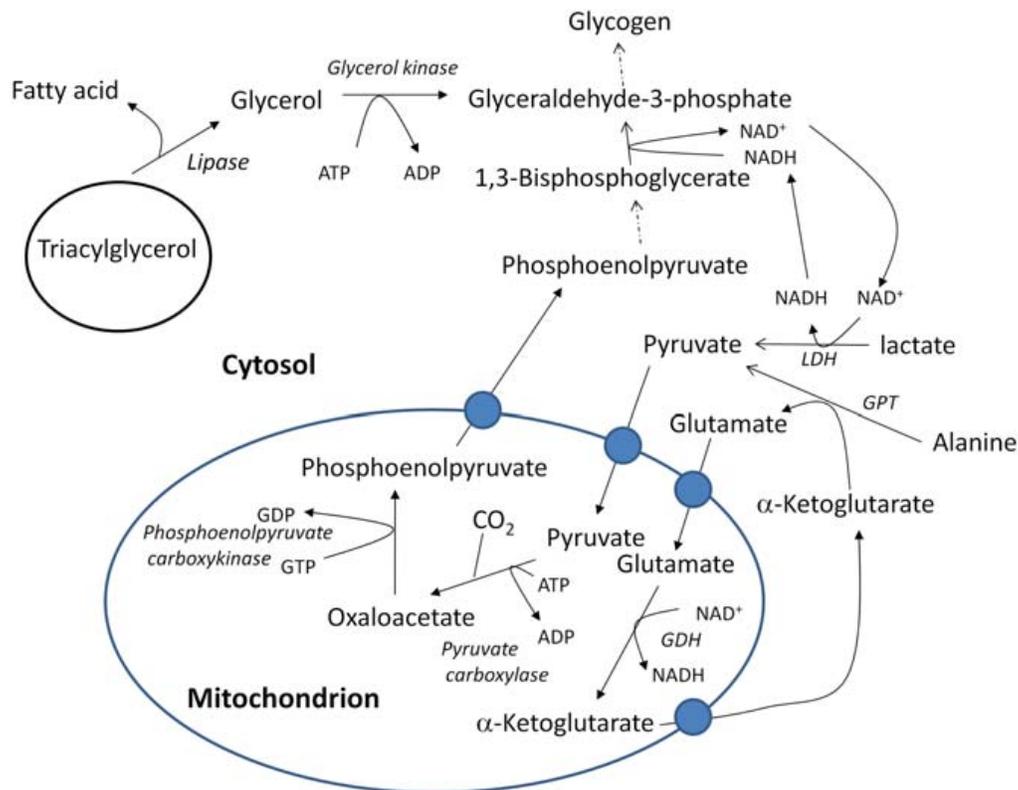


Figure 7.1. Pathways for gluconeogenesis from glycerol, lactate, or alanine in chondrichthyan liver. Enzymes are indicated in italics. See text for detailed description of the pathways. Some intermediates and enzymes have been omitted for clarity. GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; GPT, glutamate pyruvate transaminase.

PEPCK is undetectable in red and white muscle of *L. erinacea* (Moon and Mommsen, 1987) and PC is also low in red muscle (Chamberlin and Ballantyne, 1992; Crabtree et al., 1972; Opie and Newsholme, 1967). Glycogen recovery in white muscle following exhaustive exercise is slow (Richards et al., 2003). Since most lactate produced in white muscle during exhaustive exercise is not released or oxidized it must be converted to glycogen by a reversal of PK as in teleost fish (Moyes et al., 1992).

6. NITROGEN METABOLISM

Chondrichthyan must be more nitrogen-limited than any other vertebrate group with the possible exception of the coelacanth. The nitrogen metabolism of most chondrichthyan is dominated by the need to

continuously maintain high levels of urea in all body fluid compartments. Several rationales have been put forward to explain the selection of urea as the main organic solute. Urea is the ideal solute because it is small and uncharged and has a rate of diffusion closest to that of water of any naturally occurring metabolite (Ballantyne and Chamberlin, 1988; Ballantyne and Fraser, 2013). Other factors make urea a wise choice. For example urea solutions help in increasing buoyancy (Withers et al., 1994) and have antibacterial properties (Weinstein, 1947). In some conditions, the non-osmotic properties of urea seem to be more important than the osmotic role because many species in freshwater retain urea at quite high levels in spite of the lack of osmotic or energetic sense to this strategy (Ballantyne and Fraser, 2013). It has been suggested (Ballantyne and Fraser, 2013) that this is due to the apparent requirement for urea by some proteins (Yancey and Somero, 1978).

The nitrogen quotient of fed elasmobranchs is high, which indicated a reliance on amino acids as an energy source (Speers-Roesch and Treberg, 2010). Nitrogen is conserved by a lack of postprandial increase in nitrogen excretion in elasmobranchs compared to teleosts (Wood et al., 2007). Plasma ammonia levels in elasmobranchs rise slightly post feeding (Wood et al., 2010, 2005; Kajimura et al., 2006; Bucking and Wood, 2008) but less than in teleost fish. The N/O₂ ratio drops after feeding in elasmobranchs unlike the situation in all other aquatic vertebrates (Wood et al., 2007). Ammonia excretion rates are negligible during prolonged fasting in *S. acanthias* (Kajimura et al., 2008). Taken together these observations indicate a substantial need and capacity to conserve nitrogen for urea synthesis. See Chapter 5 for more detail regarding nitrogenous wastes.

6.1. Amino Acid Metabolism

Amino acids are important in all animals as intracellular solutes, neurotransmitters, oxidative substrates and as components of proteins. Glutamine is the central amino acid in the metabolism of chondrichthyans as it is in many vertebrates but for completely different reasons. In most higher vertebrates glutamine is the most abundant amino acid in the plasma, produced in muscle for export to other tissues where it is used as a nitrogen source and oxidative substrate. In elasmobranchs the levels are among the lowest and this has been attributed to the need for glutamine for urea synthesis (see Table 7.2; also see Ballantyne, 2001 for comparisons).

High activities of glutaminase in the red muscle and heart of elasmobranchs have been attributed to the use of this amino acid for oxidation (Chamberlin and Ballantyne, 1992). The occurrence of malic enzyme in red muscle mitochondria provides a mechanism whereby

Table 7.2
Metabolite concentrations (mM) in tissues of *L. erinacea*

| Metabolite | Plasma | RBCs | Liver | Muscle | Heart | Kidney | Brain | Stomach | Intestine |
|-----------------|-------------------|-------------------|---|--|-------------------|------------------|--------------------|-------------------------|-------------------------|
| Glucose | 2.8 ^a | | 3.70±0.36 ^b | 0.62±0.13 ^b | | | | | |
| Lactate | 1.52 ^a | | | | | | | | |
| Urea | 358 ^c | | 309±53 ^b | 417±14 ^b 398 ^d | 280 ^c | 320 ^b | 295 ^e | 142.0±25.2 ^f | 375.5±21.7 ^f |
| TMAO | | | 40±6 ^b | 49±2 ^b | | 19 ^h | | | |
| Ammonia | 0.37 ^c | 7.54 ^c | | 4.84 ^c | | | 6.97 ^e | | |
| Aspartate | 0.01 ^c | 0.54 ^c | | 0.75 ^c | | | 4.78 ^e | | |
| Hydroxyproline | | | | | | | | | |
| Threonine | 0.42 ^c | 0.73 ^c | 0.84 ^e | 0.94 ^c | 0.35 ^c | | 0.32 ^e | | |
| Serine | 0.26 ^c | 0.74 ^c | 0.56±0.21 ^b 0.54 ^e | 2.73±0.80 ^b 1.29 ^c | 0.21 ^c | | 1.21 ^e | | |
| Asparagine | 0.06 ^c | | | | | | | | |
| Glutamate | | 3.61 ^c | 4.45 ^e | 0.87 ^c | 3.02 ^c | | 16.2 ^e | | |
| Glutamine | 0.01 ^c | | 2.1±0.3 ^b 0.99 ^e | 2.71±1.1 ^b | | | 1.27 ^e | | |
| Sarcosine | 0.01 ^c | | 0.37±0.08 ^b | 31.67±7.47 ^b 44.1 ^c | 0.03 ^c | | | | |
| α-aminoadipate | | | | | | | | | |
| Proline | | 0.42 ^c | 0.20±0.13 ^b | 1.45±0.46 ^b 2.18 ^c | 0.09 ^c | | 0.17 ^e | | |
| Glycine | 0.07 ^c | 4.71 ^c | 0.74±0.2 ^b 0.82 ^e | 2.01±1.1 ^b 1.83 ^c | 0.25 ^c | | 7.34 ^e | | |
| L-alanine | 0.32 ^c | 2.21 ^c | 3.52 ^e | 1.96 ^c | 0.27 ^c | | 0.60 ^e | | |
| Citrulline | 0.01 ^c | | | 0.05 ^c | 0.03 ^c | | 0.027 ^c | | |
| α-aminobutyrate | 0.04 ^c | 0.92 ^c | | 0.05 ^c | | | | | |
| Valine | 0.56 ^c | 0.47 ^c | | 0.33 ^c | 0.17 ^c | | 0.17 ^e | | |
| Cystine | 0.04 ^c | 0.05 ^c | | | | | 0.061 ^e | | |
| Cysteine | | | | 0.01 ^c | | | | | |

(Continued)

Table 7.2 (Continued)

| Metabolite | Plasma | RBCs | Liver | Muscle | Heart | Kidney | Brain | Stomach | Intestine |
|---------------------------|-------------------|-------------------|-----------------------|-------------------------|-------------------|--------|--------------------|---------|-----------|
| Methionine | 0.05 ^c | 0.18 ^c | | 0.12 ^c | 0.03 ^c | | 0.125 ^c | | |
| Cystathionine | 0.05 ^c | 0.05 ^c | | 0.07 ^c | | | | | |
| Isoleucine | 0.24 ^c | 0.17 ^c | 0.16 ^g | 0.21 ^c | 0.11 ^c | | 0.064 ^e | | |
| Leucine | 0.49 ^c | 0.37 ^c | 0.3 ^g | 0.39 ^c | 0.18 ^c | | 0.134 ^e | | |
| Tyrosine | 0.05 ^c | 0.03 ^c | 0.09 ^g | 0.16 ^c | | | 0.075 ^e | | |
| Phenylalanine | 0.08 ^c | 0.05 ^c | 0.08 ^g | 0.10 ^c | 0.02 ^c | | 0.066 ^e | | |
| β-alanine | 0.03 ^c | 50.6 ^c | 1.9±0.5 ^b | 32.8±7.8 ^b | 0.72 ^c | | 1.13 ^c | | |
| γ-aminobutyrate (GABA) | | 1.23 ^c | | 40.7 ^c | | | 4.56 ^e | | |
| Creatinine | | | | 19.8 ^c | 3.07 ^c | | 7.57 ^e | | |
| Creatine | | | 4.1±0.6 ^b | 40.54±2.89 ^b | | | | | |
| Ornithine | | | | 0.16 ^c | 0.05 ^c | | 0.043 ^e | | |
| Lysine | 0.08 ^c | | | 0.39 ^c | 0.02 ^c | | 0.076 ^e | | |
| Histidine | 0.21 ^c | 0.09 ^c | | 0.3 ^c | 0.07 ^c | | 0.736 ^e | | |
| Carnosine | 0.09 ^c | | | 0.06 ^c | | | | | |
| Anserine | | | | | | | | | |
| Taurine | 0.13 ^c | | 50.8±3.8 ^b | 21.8±6.0 ^b | 48.7 ^c | | 49.0 ^e | | |
| Tryptophan | | | | | | | | | |
| Arginine | 0.11 ^c | | | 0.24 ^c | 0.01 ^c | | 0.051 ^e | | |
| Glutathione | | | | | | | | | |
| Betaine | | | 40.4±3.2 ^b | 3.87±1.38 ^b | | | | | |
| Myoinositol | | | 9.0±2.0 ^b | 0.42±0.06 ^b | | | | | |
| Glycerophosphorylcholine | | | 6.9±1.9 ^b | 0.88±0.19 ^b | | | | | |

^aGrant et al., 1969

^bSteele et al., 2005

^cBoyd et al., 1977

^dForster and Goldstein, 1976

^eForster et al., 1978

^fAnderson et al., 2010

^gBallatori and Boyer, 1988

^hMorgan et al., 2003a.

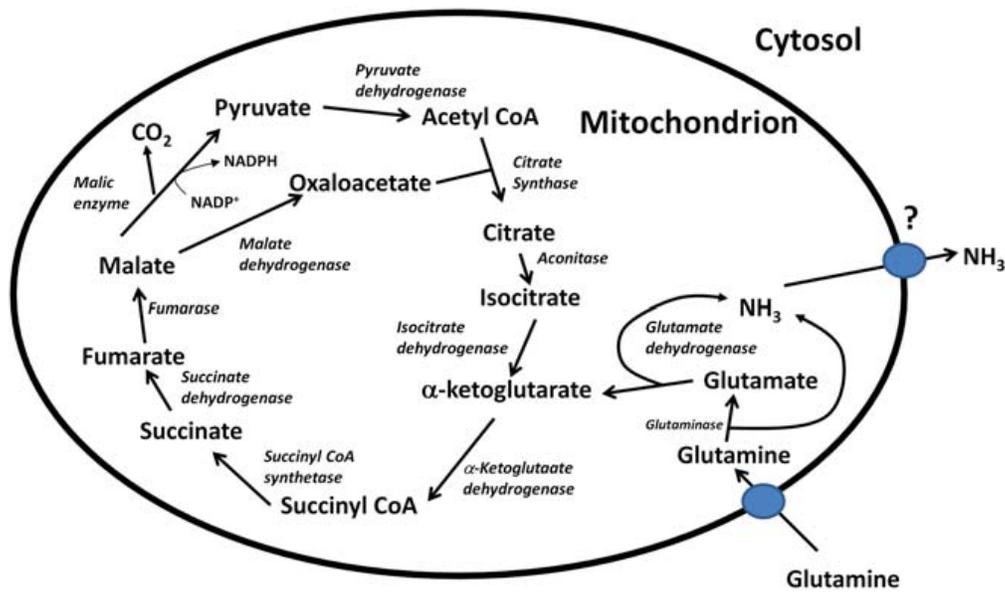


Figure 7.2. Pathway for the oxidation of glutamine in muscle and some other chondrichthyan tissues. Enzymes are indicated in italics. Some intermediates have been omitted for clarity. See text for a detailed description of the pathway.

glutamine can be oxidized without the need for other substrates (Fig. 7.2), which is similar to the pathway used in insect flight muscles that oxidize proline. Glutamine enters the mitochondria on an uncharacterized carrier and is converted to glutamate via glutaminase. Glutamate then is converted to α KG by glutamate dehydrogenase and enters the tricarboxylic acid cycle. As the cycle proceeds half of the malate produced by fumarase is converted to pyruvate via malic enzyme (NADP-dependent) and the rest is converted to oxaloacetate via malate dehydrogenase. The pyruvate is converted to acetyl CoA via pyruvate dehydrogenase and combines with oxaloacetate and continues through Krebs cycle. Thus both 2- and 4-carbon intermediates are produced to allow the TCA cycle to operate without input of carbon compounds from other pathways. The mechanism for the efflux of NH_3 from the mitochondria is not known.

Although isolated mitochondria have been demonstrated to oxidize glutamine (Chamberlin and Ballantyne, 1992) the energetic importance of this process has not been demonstrated. Indeed the role of amino acids during and following exhausting exercise is minimal based on the lack of changes in plasma alanine and total ammonia levels (Richards et al., 2003). The energetic role of glutamine oxidation via glutaminase needs to be revisited. It would seem wasteful to oxidize the amino acid that is most in demand for urea synthesis and other functions. I propose another function for muscle and heart glutaminase (Fig. 7.3). High levels of both glutamine

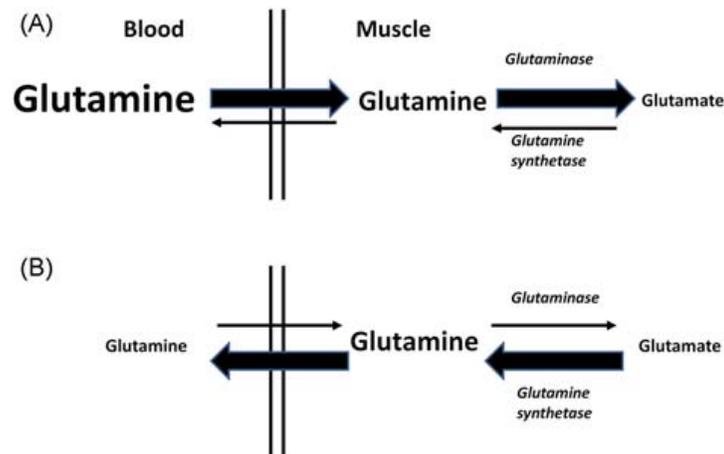


Figure 7.3. Proposed glutamine substrate cycle in muscle and blood of a chondrichthyan. (A) high blood glutamine (B) low blood glutamine. See text for a detailed description of the process.

synthetase (GS) and glutaminase in the same tissue may imply a regulatory substrate cycle for increasing the sensitivity of regulation of glutamine synthesis (Fell, 1997; Newsholme and Crabtree, 1976). Such a cycle has been proposed for avian muscle where high activities of GS and glutaminase also co-occur (Parry-Billings and Newsholme, 1991). Such a cycle in elasmobranchs could be an important mechanism for stabilizing glutamine levels in the face of large demand by liver for urea synthesis. If blood glutamine levels rise there would be an increased uptake of glutamine by muscle that would be taken by glutaminase and converted to glutamate for oxidation (Fig. 7.3). If glutamine levels in the blood fall there would be an increased release of glutamine from the muscle as the glutamine synthetase reaction predominates (Fig. 7.3). Thus glutamine oxidation in muscle may only occur if plasma levels of glutamine are high and in surplus. Glutamine synthetase is upregulated in rectal gland during fasting (Deck et al., 2013), perhaps to improve glutamine synthesis and stabilize plasma glutamine levels.

Glutamine synthetase exists in two forms coded by a single gene in elasmobranchs (Campbell and Anderson, 1991; Laud and Campbell, 1994). The mitochondrial form occurs in liver and kidney and the cytosolic isoform is found in brain and spleen (Laud and Campbell, 1994). Interestingly, two glutamine synthetase genes have been found in *Callorhincus millii* (Takagi et al., 2012). The GSs have been identified as a brain, liver, kidney form (GS1) and a muscle form GS2 (Takagi et al., 2012). The only known function of glutamine synthetase in elasmobranch liver is to provide glutamine for the urea cycle. The K_m for glutamate is high (11 mM)

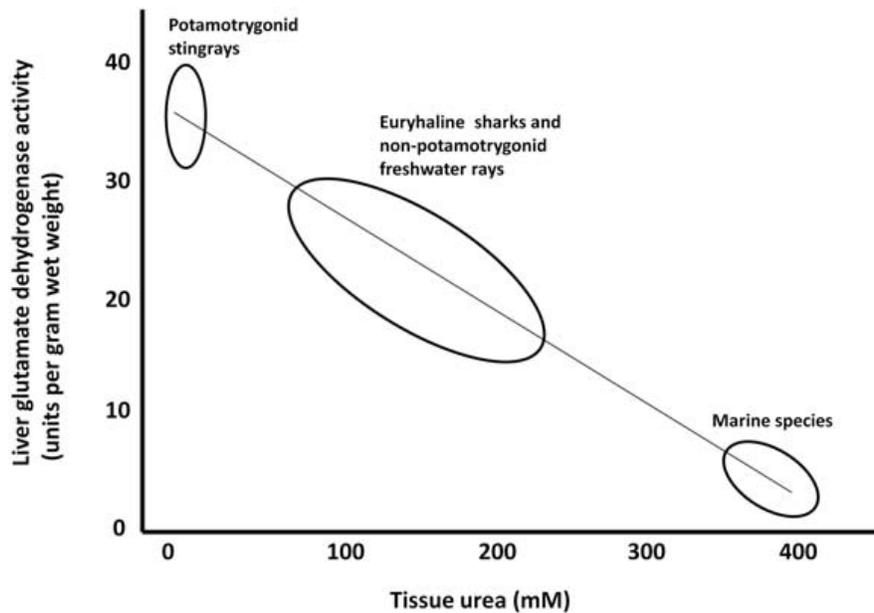


Figure 7.4. Relationship between liver glutamate dehydrogenase activity and tissue urea levels. Based on [Speers-Roesch et al. \(2006a\)](#).

([Shankar and Anderson, 1985](#)) and the enzyme is mitochondrial ([Casey and Anderson, 1982](#)). The K_m for ammonia of glutamine synthetase is very low (15 μM) ([Shankar and Anderson, 1985](#)) indicating the capacity to scavenge ammonia at very low levels and this may explain the very low plasma ammonia levels ([Wood et al., 2010](#)). Inhibition of glutamine synthetase with methionine sulfoximine did not significantly alter ammonia excretion in the kidney of *S. acanthias* ([Kormanik et al., 2000](#)). It is not known if there are isoforms of glutaminase in chondrichthyans.

Glutamate dehydrogenase (GDH) is usually associated with the oxidation of amino acids. Levels of GDH in the liver of different chondrichthyan species vary depending on the need for nitrogen for urea synthesis ([Fig. 7.4](#)). The highest activities of GDH occur in the freshwater Potamotrygonids that do not accumulate urea. The lowest levels occur in marine species where reduced amino acid oxidation ensures most glutamate is available for the synthesis of glutamine via glutamine synthetase. (See Chapter 5 for further discussion).

Ridding the cell of ammonia after amino acid oxidation (when it occurs) requires special carriers. In mammals aquaporin 8 has been identified as the ammonia carrier in mitochondria of several tissues ([Soria et al., 2010](#)). Although at least 5 isoforms of aquaporin exist in elasmobranchs ([Cutler, 2007](#)), aquaporin 8 has not yet been identified although it has been found in

teleosts (Engelund et al., 2013). In its absence another aquaporin may perform the role of transporting ammonia. One would expect a sophisticated array of ammonia and amino acid carriers in chondrichthyan cells and organelles to funnel nitrogen to the liver for urea synthesis but almost nothing is known of the transport of ammonia or amino acids into or out of cells in chondrichthyans.

6.2. Role of Amino Acids in Cell Volume Regulation

Although marine chondrichthyans are osmoconformers, amino acids make up only about 20% of the intracellular osmolarity in elasmobranchs (Boyd et al., 1977; Forster and Goldstein, 1976; Perlman and Goldstein, 1988). This is lower than that of osmoconforming marine invertebrates (>40%) due to the use of urea and methylamines as organic solutes (Yancey et al., 1982). Intracellular levels of amino acids do change in response to salinity changes (Boyd et al., 1977).

Taurine is the main organic solute used by most cells for cell volume regulation, selected presumably for its lack of involvement in other metabolic processes and protein synthesis. In *L. erinacea* liver, taurine and betaine are the main osmotically important amino acids while in muscle taurine, sarcosine and β -alanine are important (Table 7.2). In heart and brain, taurine are the main amino acids and in RBCs it is β -alanine (Table 7.2). Aside from the fact that all of the osmotically important amino acids are not found in protein, the basis for the differences in major amino acids in different tissues has not been explained. Elasmobranchs cannot synthesize taurine (King et al., 1980b) so must obtain it from the diet. This is similar to the situation in other carnivores.

More than one type of transporter/carrier is involved in cell volume regulation with organic osmolytes. Cell volume regulation in skate erythrocytes involves sodium-independent volume-sensitive organic osmolyte channels with wide specificity (Renfro, 2005). These channels have been identified as anion exchange proteins (sometimes called band 3) and three forms have been identified in skate erythrocytes (Guizouarn et al., 2003). Under isosmotic conditions the channels are sequestered in cytosolic cholesterol-rich rafts and these are mobilized into the plasma membrane under hypoosmotic conditions to allow taurine efflux to reduce cell volume. Cell swelling causes the channel, normally a dimeric protein in the plasma membrane to form a tetramer (Musch et al., 1999). Tyrosine phosphorylation is involved in regulation (Koomoa et al., 2005) with an activation of two of the four tyrosine kinases found in skate erythrocytes: specifically p72^{syk} and p56^{lyn} (Musch et al., 1999). Organic osmolyte channels have been detected in erythrocytes, hepatocytes, and heart cells of elasmobranchs

(Perlman and Goldstein, 1999) but likely occur in most cells types. The mechanism of taurine transport differs between RBCs and hepatocytes (Davis-Amaral et al., 1997).

Another way of disposing of osmotically important amino acids is by oxidation. Enhanced oxidation of amino acids such as β -alanine has been reported during hypoosmotic conditions (Leech and Goldstein, 1983; King et al., 1980a) although oxidation by isolated mitochondria is insensitive to changing osmotic conditions (Moyes et al., 1986a). Sarcosine oxidation by both isolated hepatocytes and mitochondria increases at lower osmolarities (Ballantyne et al., 1986). The importance of this in the intact animal is not known. (See Chapter 5 for further discussion).

6.3. Purine Nucleotide Cycle

Although it is assumed to function in elasmobranchs, the only enzyme of the purine nucleotide cycle to be demonstrated is AMP deaminase (Thebault et al., 2005). AMP deaminase activity is higher in white muscle than red and much higher in *L. erinacea* muscles than in those of *Scyliorhinus canicula* (Thebault et al., 2005). AMP deaminase is regulated by energy charge as in other vertebrates (Izem et al., 1993) and there is evidence that in exhaustive exercise reduced adenylate levels are reduced by deamination (Richards et al., 2003). High levels of AMP deaminase have been reported for whole blood (Raffin and Leray, 1980; Raffin, 1983) and gill (Raffin and Leray, 1980). Higher activities of AMP deaminase in the erythrocytes of elasmobranchs distinguishes them from teleosts (Raffin and Leray, 1980) and merits further investigation. Although a role for AMP deaminase in urea formation has been discounted in mammals (Nissim et al., 1992) its function beyond the purine nucleotide cycle in chondrichthyan should be examined.

6.4. Pyrimidine Pathway

De novo pyrimidine synthesis requires glutamine as a nitrogen source and this may constrain where it can occur. The liver lacks CPSase II and aspartate carbamoyl transferase precluding its role in pyrimidine synthesis (Anderson, 1989). This may be due to the overriding need for urea synthesis in liver, which would lead to a competition for glutamine. A cytosolic CPSase II and a cytosolic glutamine synthetase and the other enzymes that function in pyrimidine synthesis have been identified in spleen and testis (Anderson, 1989). These enzymes are not associated into a multienzyme complex as occurs in higher animals (Anderson, 1989). There is no evidence

that the pyrimidine salvage pathway involving thymidine kinase and thymidilate kinase occurs in elasmobranchs.

6.5. Urea Synthesis

The pathway and kinetics of the enzymes in the elasmobranch urea cycle are well understood largely due to the work of Paul Anderson and colleagues. Less is known of the mechanisms of regulation. The compartmentalization of urea synthesis in elasmobranch liver (Fig. 7.5) differs from that of mammals and lungfish. The main difference is the localization of arginase, which is mitochondrial in elasmobranchs and cytosolic in mammals (Anderson, 1991). Additionally, the enzyme carbamoyl phosphate synthase (CPS) of elasmobranchs uses glutamine as the nitrogen donor (CPSIII) (Anderson, 1991) whereas the mammalian enzyme uses ammonia directly.

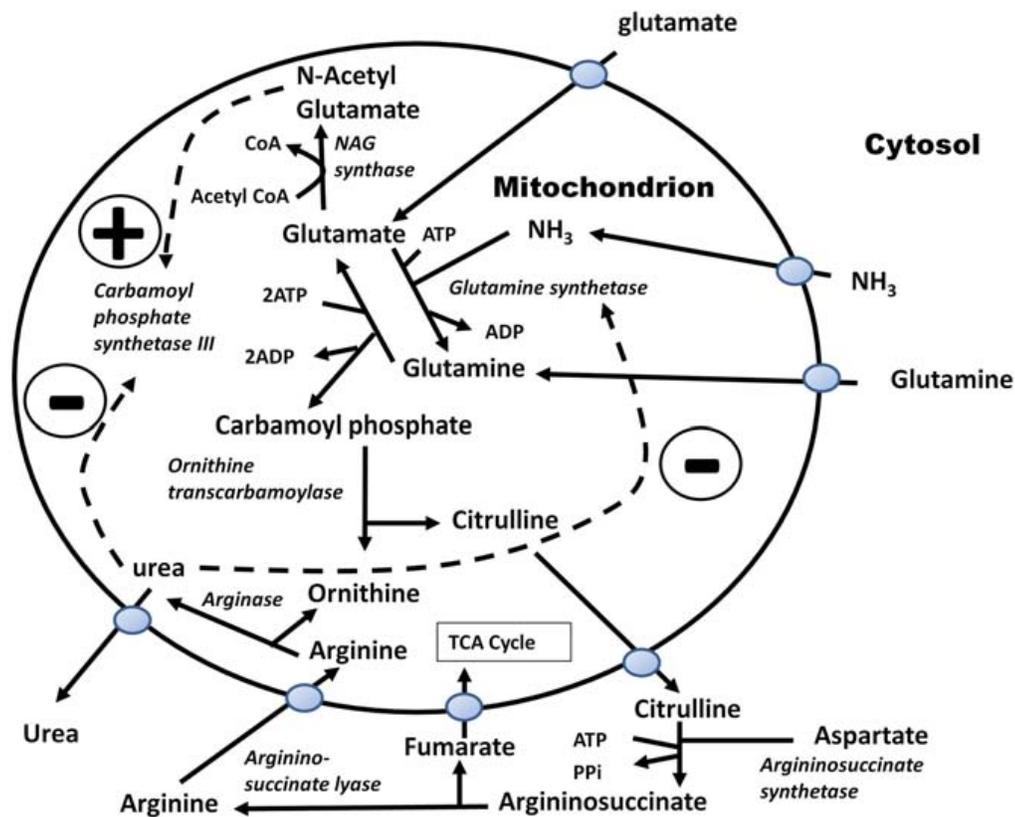


Figure 7.5. The compartmentation of the urea cycle in the chondrichthyan liver. Enzymes are indicated in italics. Some intermediates have been omitted for clarity. See the text for a detailed description of the pathway. NAG, N-acetyl glutamate; TCA cycle, tricarboxylic acid cycle.

CPSase III is mitochondrial and is activated by N-acetylglutamate. The K_m for glutamine is low (160 μ M) (Anderson, 1981) perhaps due to the need to scavenge glutamine against low circulating levels. The mitochondrial carriers for glutamate, glutamine, citrulline, fumarate, and arginine have not been identified or characterized.

The promoter region of CPSIII for *S. acanthias* has been sequenced and found to contain two regions for binding C-EBP-related proteins as is known for the mammalian CPSase I for tissue specific expression in the liver (Hong et al., 1996). Additionally, two heat-shock protein binding regions were found as occurs in mammals perhaps indicating a role of stress (perhaps salinity challenge) in regulating urea synthesis (Hong et al., 1996).

A single CPSase III and ornithine transcarbamylase has been found in *Callorhincus millii* (Takagi et al., 2012). Studies of the expression of urea cycle enzymes in *C. millii* indicate urea synthesis predominates in liver but kidney and muscle may also contribute (Takagi et al., 2012). Extrahepatic urea synthesis may also occur in muscle of *L. erinacea* (Steele et al., 2005) and intestine of *S. acanthias* (Kajimura et al., 2006). The function of extrahepatic urea synthesis needs to be examined further.

Arginase catalyzes the last reaction in the urea cycle. There are two genes for arginases (ARG2 and ARG1) in *C. millii* with the ARG2 involved in the urea cycle (Takagi et al., 2012). Oddly, ARG1 is nonfunctional so effectively only one isoform of the enzyme exists (Takagi et al., 2012). In chondrichthyans, arginase is mitochondrial and has a K_m for arginine of 1.2 mM about 10-fold lower than that of teleost fish (Jenkinson et al., 1996). Higher vertebrates also have a cytosolic arginase (arginase I) derived from the ancestral mitochondrial form (arginase II) (Srivastava and Ratha, 2010). Intact elasmobranch liver mitochondrial arginase has different kinetics than that of the isolated enzyme (Barbosa et al., 2005).

Regulation of urea synthesis (Fig. 7.5) may involve N-acetyl glutamate (NAG). NAG levels are higher in liver of elasmobranchs and other groups using CPSase III as the mechanism for urea synthesis (Julsrud et al., 1998). It is not known if arginine activates NAG synthase in chondrichthyans as occurs in mammals (Haskins et al., 2008). Urea may also regulate the activity of the urea cycle since CPSase III and glutamine synthetase are inhibited by physiological levels of urea (Fig. 7.5) (Anderson, 2001).

A variety of physiological and environmental states impact urea synthesis. Urea synthesis declines with starvation or low protein intake in *Scyliorhinus canicula* but urea clearance is also reduced to maintain urea levels (Armour et al., 1993). In some species such as *Poroderma africanum*, during starvation, urea levels fall by about 20% causing a concomitant fall in osmolarity with the net effect of shifting the fish from a slightly hypertonic state to a slightly hypotonic state (Haywood, 1973). By contrast

in *S. acanthias* plasma urea levels are stable for at least 2 months of starvation although rates of urea synthesis were not determined (Wood et al., 2010). There is a postprandial increase in urea cycle enzyme activity in liver and white muscle (Kajimura et al., 2006) and plasma urea levels rise in *S. acanthias* (Wood et al., 2010). Environmental salinity impacts urea synthesis. Hepatocytes isolated from *C. leucas* adapted to seawater had urea synthesis rates 2.7 fold higher than those isolated from freshwater-adapted fish (Anderson et al., 2005). Urea synthesis in dilute environments is lower in *L. erinacea* (Goldstein and Forster, 1971), and *Hemiscyllium plagiosum* (Wong and Chan, 1977) but not in *Negaprion brevirostris* (Goldstein et al., 1968) and *Scyliorhinus canicula* (Alexander et al., 1968). The basis for these interspecies differences needs to be investigated further.

A high affinity energy-dependent, phloretin-sensitive urea transporter with a K_m for urea of 0.34 mM has been identified in liver mitochondria of *L. erinacea* (Rodela et al., 2008). As the site of urea synthesis, liver mitochondria must be equipped to allow urea to move out of the matrix into the cytosol. Such a low K_m is difficult to interpret since urea levels in the mitochondrial matrix are unknown. It has been assumed that urea levels in the mitochondrial matrix are very high, similar to those of all other fluid compartments (Ballantyne and Moon, 1986) but this may not be the case. Since urea produced in the liver needs to be delivered to the mitochondrial matrix of other tissues one would assume the appropriate carriers are present in the plasma membranes of all cells but this has not been established.

Permeability of epithelia to urea plays an important role in controlling the loss of urea. Reduced permeability due to structural and transport features of basolateral membranes in three of the main sites of loss of urea – the gills (Fines et al., 2001), kidney (Morgan et al., 2003b), and rectal gland (Zeidel et al., 2005) – have been reported. In gill, a sodium-linked urea transporter and high cholesterol content have been identified as possible mechanisms for controlling urea losses at that tissue (Fines et al., 2001). Recovery of urea from the filtered plasma in the kidney plays a major role in the nitrogen economy of elasmobranchs. The kidney has two known carriers in *L. erinacea*. One is a high affinity (0.7 mM) sodium-dependent carrier (Morgan et al., 2003b) for recovering filtered urea and the other is a non-saturable carrier (Morgan et al., 2003b). Both carriers are down-regulated at lower salinities (Morgan et al., 2003a).

Low levels of urea have been reported in some parts of the gastrointestinal tract in three species of elasmobranchs (Anderson et al., 2010). The lowest levels were in the colon and stomach with the intestine having levels similar to that of the plasma. This plays a role in recovery of urea from the GI tract. Urea uptake via an active transport mechanism

occurs primarily in the intestine at rates comparable to those of the gills and kidney and the rate increases with feeding (Liew et al., 2013). (See also Chapter 5 for further discussion).

6.6. Methylamines and β -Amino Acids

Methylamines are accumulated in elasmobranch tissues as part of a strategy to counteract the disruptive effects of urea on the structure of some proteins (Yancey and Somero, 1979). Urea/methylamine ratios vary from tissue to tissue (Bedford et al., 1998a) but nothing is known of how this is coordinated. Trimethylamine N-oxide (TMAO), betaine, and sarcosine are the main methylamines accumulated in elasmobranchs. The capacity for synthesis of TMAO has a phylogenetic basis with Chimeriformes, Rajiformes, and Squaliformes lacking this capacity (Treberg et al., 2006b). *Chiloscyllium punctatum* (Treberg et al., 2006b) and *Ginglymostoma cirratum* (Goldstein and Dewitt-Harley, 1973; Goldstein and Funkhouser, 1972) from the Order Orectolobiformes and *Negaprion brevirostris* (Goldstein and Dewitt-Harley, 1973) and *Mustelus californicus* (Baker et al., 1963) from Carcharhiniformes can synthesize TMAO. TMAO levels rise post feeding in *S. acanthias* and remain stable during 2 months of fasting (Wood et al., 2010). TMAO levels remain stable during 45 days of fasting in the skate, *L. ocellata* (Treberg and Driedzic, 2006). Since these 2 species cannot synthesize TMAO (Treberg and Driedzic, 2006) the capacity to maintain plasma levels has been attributed to improved retention (Treberg and Driedzic, 2006) and the ability to use intracellular TMAO as muscle cells shrink (Wood et al., 2010).

Tissue levels of specific methylamines vary with TMAO being found in high levels in most tissues of *L. ocellata* (Treberg and Driedzic, 2007). In the same species, betaine is high in white muscle and heart while sarcosine is high only in white muscle and brain (Treberg and Driedzic, 2007). Betaine is not high in the tissues where it is synthesized implying intertissue transport must occur. In holocephalans, betaine replaces TMAO as the main counteracting solute (Bedford et al., 1998b). The basis for the differences in methylamine profiles in different tissues has not been established.

As with urea, methylamine levels need to respond to salinity challenges. TMAO levels change with salinity in *L. ocellata* but sarcosine and betaine do not (Treberg and Driedzic, 2007). Freshwater species that retain urea have methylamines and β -amino acids while the Potamotrygonids that accumulate no urea have only β -amino acids (Treberg et al., 2006b).

The established and potential pathways involved in the synthesis of methylamines and β -amino acids are described in Chapter 4. TMAO synthesis is microsomal with trimethylamine (TMA) being converted to

TMAO via TMAO oxidase. TMA may be synthesized from betaine as occurs in a marine teleost, by an, as yet unknown, pathway (Charest et al., 1988). Betaine can be synthesized from choline via choline dehydrogenase and betaine aldehyde dehydrogenase with betaine aldehyde as an intermediate. Betaine aldehyde dehydrogenase and choline dehydrogenase are mitochondrial (Treberg and Driedzic, 2007). Sarcosine can be synthesized from dimethylglycine via dimethylglycine dehydrogenase. Dimethylglycine in turn can be synthesized from betaine via a cytosolic betaine homocysteine transmethylase. Sarcosine is degraded to glycine via mitochondrial sarcosine dehydrogenase. Earlier descriptions of the dimethylglycine dehydrogenase and sarcosine dehydrogenase reactions in elasmobranchs gave formaldehyde as a product but it is more likely that 5,10 methylene tetrahydrofolate is a product for both reactions as in rat liver (Porter et al., 1986). Liver and kidney are the main sites of betaine synthesis in *L. ocellata* (Treberg and Driedzic, 2007) but the tissue sites of synthesis of some other compounds remains to be established. Dietary betaine is important in maintaining high levels of tissue betaine even in species where betaine synthesis occurs (Treberg and Driedzic, 2007). Adaptation to higher salinity does not change tissue betaine levels (Treberg and Driedzic, 2007).

Methylamines become increasingly important as solutes with increasing depth due to their role in protecting protein function at high pressures. Increasing pressure would exacerbate the disruptive effects of urea on protein structure while methylamines are thought to counteract the disruptive effects of pressure (Samerotte et al., 2007; Kelly and Yancey, 1999). Because marine elasmobranchs are osmoconformers, a decrease in one solute must be accompanied by an increase in another. Urea/TMAO ratios range from 1.8 in white muscle to 6.02 in rectal gland of *S. acanthias* while in the deep-sea shark *Centroscyllium fabricii* the ratio has a much narrower and lower range (0.97 vs 1.84) (Treberg and Driedzic, 2002). It has been suggested that the inability to reduce urea and increase methylamines further limits their capacity to colonize the deeper waters accessible to teleosts (Laxson et al., 2011). Nothing is known of how this is coordinated. See Chapter 4 for more details regarding the mechanism of action of methylamines.

TMAO transport in elasmobranch erythrocytes may occur by the same channel as for taurine efflux (Koomoa et al., 2001). TMAO transport across the plasma membrane of erythrocytes occurs by sodium-dependent and a sodium-independent mechanisms with the sodium-independent pathway stimulated by hypotonic conditions (Goldstein, 1999; Wilson et al., 1999). TMAO is reabsorbed in the kidney but the mechanism has not been identified (Evans et al., 2004). (See also Chapter 4 for further discussion).

6.7. Creatine Metabolism

Creatine phosphate is important as a phosphagen in exhaustive exercise (Richards et al., 2003). It occurs in two forms as in the Agnatha (Fisher and Whitt, 1978) compared to three or more in advanced vertebrates (Fisher et al., 1980). It is not known if any of the forms are mitochondrial. Creatine phosphate levels are about 4 mM in epaulette shark and shovelnose ray heart (Speers-Roesch et al., 2012a). Creatine kinase levels are higher in deep-sea elasmobranch tissues compared to similar functioning shallow water species (Treberg et al., 2003) but the basis for this is unknown.

6.8. Nitric Oxide

Nitric oxide has a range of functions in animals ranging from a role in the immune response to regulating blood flow, gut motility, and neurotransmission. Not all of these functions have been identified in chondrichthyans. Nitric oxide is synthesized from arginine via nitric oxide synthase (NOS). In mammals, there are three isoforms: a neuronal form (nNOS), an endothelial form (eNOS), and an inducible form (iNOS). Only two of these occur in chondrichthyans. NOS is found in the gut nerves of elasmobranchs (Olsson and Karila, 1995), the nerves of the olfactory epithelium of *Scyliorhinus canicula* (Ferrando et al., 2012), and in the gill of *S. acanthias* (Evans, 2000). Inducible NOS occurs in peripheral leucocytes in elasmobranchs as part of the immune response to bacteria (Walsh et al., 2006a) and a NOS identified as iNos has been reported from the genome of *S. canicula* (Gonzalez-Domenech and Munoz-Chapuli, 2010). The endothelial form has not been reported from chondrichthyans. It is absent from the dorsal aorta of *Callorhinchus millii* and the guanylyl cyclase signaling system is also absent (Jennings et al., 2007). Prostaglandins seem to fulfill the role of mediating vasodilation in elasmobranchs because they have been shown to be involved in vasodilation in the aorta of *S. acanthias* but NOS is not (Jennings et al., 2007; Evans and Gunderson, 1998).

Nitric oxide levels in brain of *H. ocellatum* increase during hypoxia although brain blood flow does not change (Renshaw and Dyson, 1999). Studies of *S. acanthias* indicate that nitric oxide is not a vasodilator in normoxia but is in hypoxia (Swenson et al., 2005). If eNOS is not found in elasmobranchs this observation implies another isoform replaces it. Adenosine does affect cerebral blood flow during normoxia (Soderstrom et al., 1999) and branchial circulation is also regulated by adenosine involving A1 and A2 receptors and is mediated by nitric oxide (Pellegrino et al., 2005).

7. LIPID AND KETONE BODY METABOLISM

The lipid metabolism of chondrichthyans is unusual in several respects. These include the array of storage lipids, the lack of use of fatty acid oxidation in red muscle and heart, and the high constitutive reliance on lipid-derived ketone bodies. Starving elasmobranchs differ from starving teleosts in that they do not appear to mobilize lipid in the early stages (Speers-Roesch and Treberg, 2010). As the earliest known vertebrates to use myelin to insulate nerves, elasmobranchs would have enhanced nerve conduction velocities that would represent improved neural function over the agnathans. Little is known of the metabolism of myelin in chondrichthyans beyond a single study (Agrawal et al., 1971). Methoxylated alkylglycerols occur in elasmobranchs and although their function has not been established they have been reported to have antibiotic properties (Magnusson and Haraldsson, 2011).

7.1. Storage Lipids

There are four main lipid storage forms in elasmobranchs: triacylglycerol (TAG), alkyldiacylglycerol (DAG), wax ester, and squalene. All do not necessarily occur together. Of these squalene is the only one that is not readily metabolized as an energy source. All are stored primarily in the liver. Lipid stored in the liver in large amounts serves two important functions. The first is as an energy reserve and source of ketone bodies to supply other tissues with oxidizable carbon. The second is as a buoyancy organ similar to the swim bladder of more advanced fishes. TAGs confer the least buoyancy per gram (density = 0.92 gm/ml) while DAGs have a lower density (0.89 gm/ml). Squalene and wax esters confer the greatest buoyant effect with a density of 0.86 gm/ml. Experimentally the proportion of DAGs to TAGs in liver has been shown to increase in *S. acanthias* when their density is increased with weights (Malins and Barone, 1970). Fine-tuning density by adjusting proportions of squalene and DAGs occurs with increasing size in deep-sea species (Wetherbee and Nichols, 2000). The turnover of TAGs in plasma is slower than that of DAG in *S. acanthias* (Sargent et al., 1972) perhaps indicating its greater role in energy metabolism in some species. It has been suggested that DAGs are conserved for buoyancy (Sargent et al., 1972). The two functions of energy reserve and buoyancy may conflict during periods of fasting associated with migration. As the lipid reserves are depleted buoyancy is reduced impacting the energetic of migration. This has been analyzed for the great white shark, *Carcharodon carcharias* (Del Raye et al., 2013). It has been suggested that in

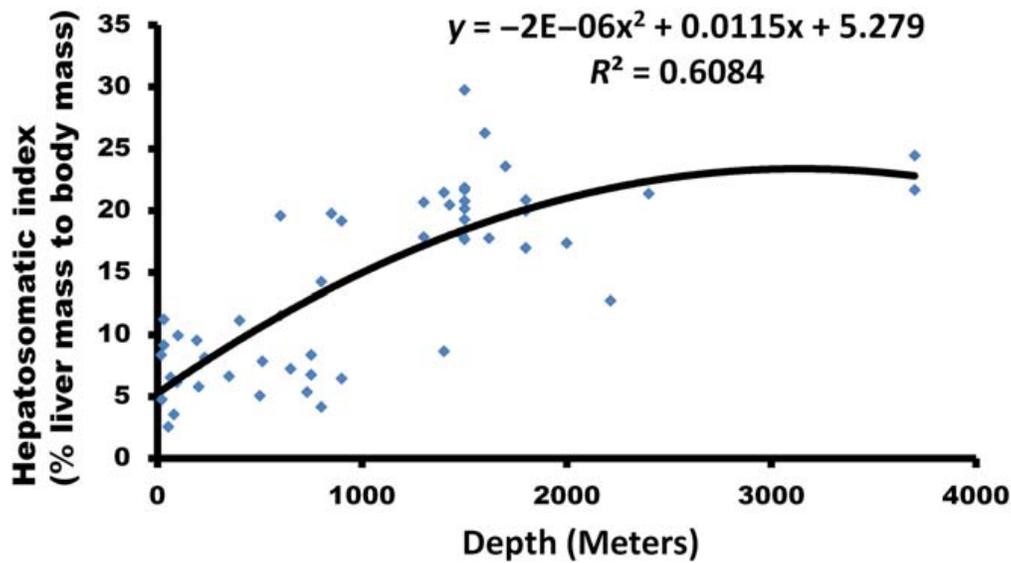


Figure 7.6. The relationship between depth of occurrence and the hepatosomatic index. Data are taken from [Bordier et al. \(1996\)](#), [Deprez et al. \(1990\)](#), [Hernandez-Perez et al. \(1997\)](#), [Van Vleet et al. \(1984\)](#), [Davidson and Cliff \(2002\)](#), [Jayasinghe et al. \(2003a\)](#), [Navarro-Garcia et al. \(2000\)](#), [Jayasinghe et al. \(2003b\)](#), [Navarro-Garcia et al. \(2004b\)](#), [Navarro-Garcia et al. \(2009\)](#), [Navarro-Garcia et al. \(2004a\)](#), and [Pethybridge et al. \(2010\)](#). Depths were taken from the site of capture where reported or from Fishbase ([Froeser and Pauly, 2015](#)).

large sharks the role of liver lipid in buoyancy is more important than its role as a lipid reserve ([Baldrige, 1972](#)). Female raggedtooth sharks, *Carcharias Taurus*, increase liver mass and store larger amounts of lipid in the liver in preparation for mating and bearing the young in utero ([Davidson and Cliff, 2011](#)). Deep water species feed less frequently and thus would need greater lipid reserves as energy stores, but may have a greater need for buoyancy to reduce energy demands for locomotion.

The size of the liver increases with depth of occurrence ([Fig. 7.6](#)) such that the hepatosomatic index (HSI) reaches a maximum at about 2000 meters and a size of 20–25% of body weight. The lipid content of the liver also increases with depth, but levels off at about 2000 meters and a content of 60–90% ([Fig. 7.7](#)). Elasmobranchs are uncommon below 2000 meters depth and do not occur below 3000 meters ([Musick and Cotton, 2014](#); [Priede et al., 2006](#)). The 2000–3000 meter limit has been suggested to be due to energetic constraints in obtaining enough food to maintain high lipid levels in the liver ([Priede et al., 2006](#)) and a high trophic level position ([Musick and Cotton, 2014](#)).

In general deepwater species have higher liver DAG content but there are exceptions ([Magnusson and Haraldsson, 2011](#)). The highest proportion of DAG occurs in a midwater shark, *Centroscymnus plunketi*, at 77%

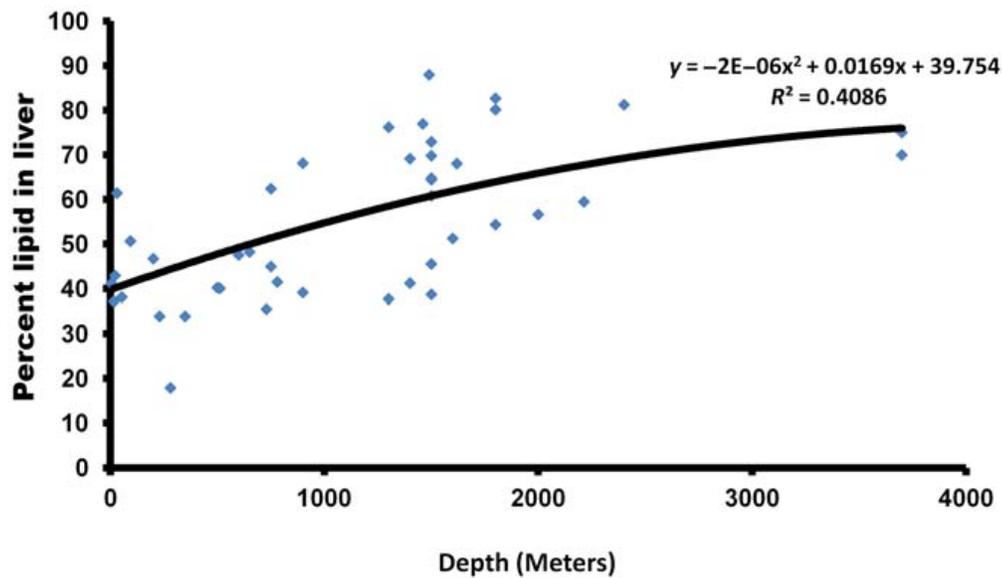


Figure 7.7. The relationship between maximal depth of occurrence and the percent lipid in the liver of elasmobranchs. Data are from [Deprez et al. \(1990\)](#), [Wetherbee and Nichols \(2000\)](#), [Hernandez-Perez et al. \(1997\)](#), [Hernandez-Perez et al. \(2002\)](#), [Hayashi and Takagi \(1981\)](#), [Jayasinghe et al. \(2003a\)](#), [Navarro-Garcia et al. \(2000\)](#), [Sargent et al. \(1973\)](#), [Jayasinghe et al. \(2003b\)](#), [Craik \(1978\)](#), [Navarro-Garcia et al. \(2004a,b, 2009\)](#), and [Pethybridge et al. \(2010\)](#). Depths were taken from the sites of capture if reported or from Fishbase ([Froeser and Pauly, 2015](#)).

([Bakes and Nichols, 1995](#)). Similarly, while squalene levels are higher in deeper water, the highest levels are reported for midwater species such as *Centrophorus moluccensis* (82% of liver lipid) ([Bakes and Nichols, 1995](#)) and *Centrophorus lusitanicus* (76% of liver lipid) ([Hernandez-Perez et al., 1997](#)). It may be that at greater depths there is not enough food available to support synthesis of large amounts of squalene. There are sexual differences in squalene with male deep-sea sharks having higher liver squalene content than females ([Hernandez-Perez et al., 2002](#)), which perhaps indicates the deposition of squalene in developing embryos. The rationale for using such large amounts of squalene in some deep seas sharks is a metabolic mystery because it cannot be used as an energy source via currently known pathways.

Within a species the content of DAG and TAG can vary considerably. For example *S. acanthias* from the North Atlantic have a 18% DAG and 81% TAG while those from the North Pacific have 45% DAG and 47% TAG ([Magnusson and Haraldsson, 2011](#)), which likely reflects dietary influences. If the Pacific population of *S. acanthias* is really *S. suckleyi* and suggested by [Ebert et al. \(2010\)](#), the above compositions

would be interspecies differences. Changing dietary lipids can influence stored lipids in muscle and liver within a relatively short time (3 weeks) (Beckmann et al., 2013).

The various storage lipids in liver contain different arrays of fatty acids. In *S. acanthias* TAGs and DAGs contain primarily saturates and monounsaturated fatty acids (Malins et al., 1965) and the fatty acids in the 2 position of triacylglycerols tend to be more polyunsaturated, with 22:n3 predominating (Brockhoff et al., 1968). Lipids stored as TAGs and DAGs in liver are more saturated than those in muscle of *S. acanthias* (Malins et al., 1965). Wax esters in *S. acanthias* serum contain more polyunsaturated fatty acids (PUFA), especially 20:5n3 and 22:6n3, while the fatty alcohols are mainly saturated (16:0) and monounsaturated (18:1n9) (Sargent et al., 1973). DAG are composed largely of saturated and monounsaturated acyl chains (Magnusson and Haraldsson, 2011).

In general, where measured, the n3/n6 ratio is different from that of teleosts in the same environment. The proportions of the two essential fatty acid types (n3 and n6) vary with tissue, environment, and diet. The n3/n6 ratio of deep-sea shark muscle is slightly lower (5.5–6.1) than that of deep-sea teleosts (6.8–7.4) (Okland et al., 2005). Great white shark muscle has a n3/n6 of 1.50 and liver 2.33 (Pethybridge et al., 2014). Tropical sharks have a low n3/n6 (range = 1.16–3.7) in liver lipids (Davidson and Cliff, 2002). Deep-sea sharks have a fivefold higher arachidonic acid content than that of teleost fish (Stoknes et al., 2004). Seasonal decreases in docosahexaenoic acid (22:6n3; DHA) content of liver TAGs occurred in winter for *Lamna ditropis* (Jayasinghe et al., 2003b). High levels of n6 fatty acids have been reported in whale shark *Rhincodon typus* and muscle of *Manta alfredi* even though the diet consisted of crustacean rich in n3 fatty acids (Couturier et al., 2013). The difference in the retention of n3 and n6 fatty acids compared to teleosts merits further investigation.

7.2. Lipid Synthesis

The two main sites of wax ester and TAG synthesis from dietary lipids are the liver and intestinal mucosa (Sargent et al., 1971, 1972; Friedberg and Greene, 1967b). Rates of synthesis of wax esters and triacylglycerols are similar and greater than that of DAGs in *S. acanthias* (Malins and Sargent, 1971; Sargent et al., 1972). Synthesis of DAGs from dietary fatty alcohols has been demonstrated in the stomach of *S. acanthias* and *L. erinacea* (Friedberg and Greene, 1967a). Subsequent transport to the liver for storage must occur but the mechanism has not been investigated. Little is known of the mechanisms of regulation of synthesis of wax esters versus TAGs or DAGs and where these occur together competition for acyl CoA implies

some compartmentation may be required. Further research in this area is needed.

Fatty acids are preferentially incorporated into TAGs compared to DAGs in *S. acanthias* (Malins, 1968) and fatty alcohols are preferentially incorporated into DAG (Malins and Sargent, 1971). The pathway for *de novo* synthesis of lipids may differ from that of other fish. While elasmobranch mitochondria can transport tricarboxylates based on oxidation of exogenously applied citrate and isocitrate by isolated liver mitochondria (Moyes et al., 1986b), the absence of cytosolic malic enzyme (Zhou et al., 1995) means NADPH must be generated by other means. A cytosolic isocitrate dehydrogenase (Saxrud et al., 1996) may provide the needed reducing equivalents. See Fig. 7.8 for the suggested pathway for *de novo* lipid synthesis in the liver of elasmobranchs. Carbon for lipid synthesis may come from carbohydrate or amino acid sources. Ultimately, pyruvate enters the mitochondria via the monocarboxylate carrier and one half is

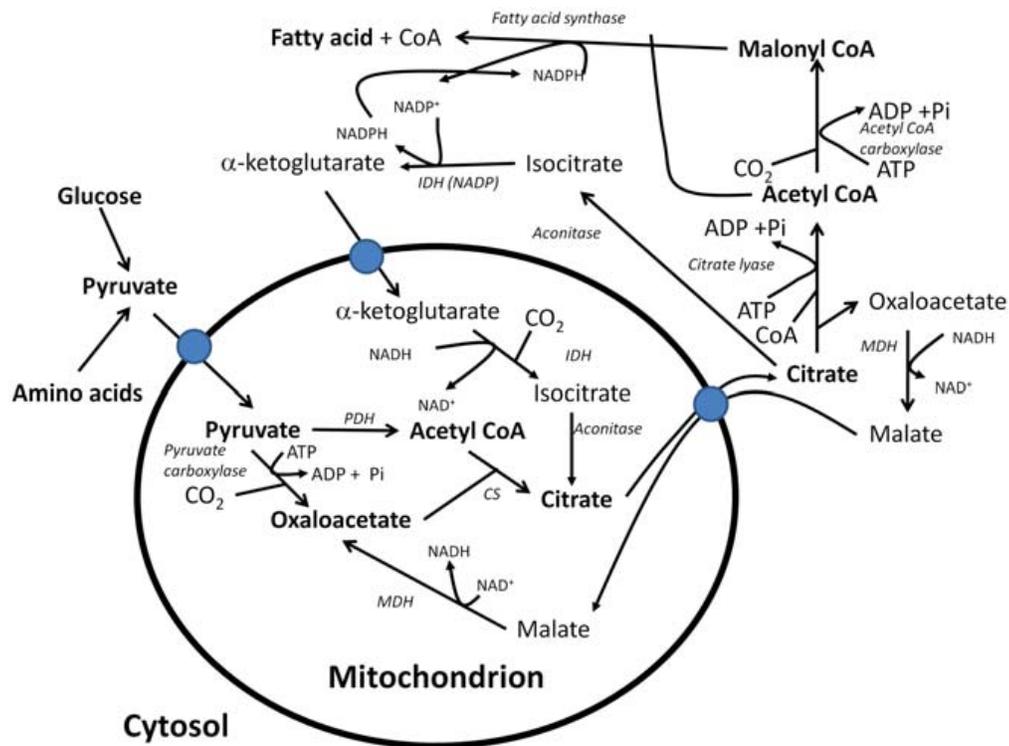


Figure 7.8. Pathway for *de novo* lipid synthesis in chondrichthyans. Intermediates in bold font indicate those involved in the flow of carbon. Enzymes are indicated in italics. Some intermediates have been omitted for clarity. IDH, NAD-dependent isocitrate dehydrogenase; IDH (NADP), NADP-dependent isocitrate dehydrogenase; PDH, pyruvate dehydrogenase; MDH, malate dehydrogenase; CS, citrate synthase. Modified from (Ballantyne, 2014).

converted to acetyl CoA via pyruvate dehydrogenase and the other half to oxaloacetate via mitochondrial pyruvate carboxylase. Acetyl CoA and oxaloacetate combine to form citrate catalyzed by citrate synthase. The citrate is transported out of the mitochondria perhaps in exchange for malate. In the cytosol the citrate is converted to both acetyl CoA via citrate lyase and isocitrate via cytosolic aconitase. Acetyl CoA is converted to malonyl CoA by acetyl CoA carboxylase, which enters the fatty acid synthase complex for successive additions of 2 carbon units to achieve the appropriate fatty-acid chain length. In the absence of cytosolic NADP-dependent malic enzyme (Zhou et al., 1995) cytosolic redox for the fatty acid synthase is balanced by a NADP-dependent isocitrate dehydrogenase (Saxrud et al., 1996). It is not known if the pentode phosphate pathway is also involved in generating NADPH for lipogenesis in chondrichthyan liver. The oxaloacetate produced in the cytosol by citrate lyase is converted to malate by cytosolic malate dehydrogenase and transported into the mitochondria in exchange for citrate as described above. Much more work is needed to validate this model. Levels of cytosolic isocitrate dehydrogenase increase in rectal gland post feeding (Dowd et al., 2008), perhaps due to the involvement in lipid synthesis.

Dietary intake of squalene is low and accumulation of large amounts in the liver is due to hepatic synthesis (Blumer, 1967; Sargent et al., 1970). Mevalonic acid is an intermediate in squalene synthesis as in other animals (Sargent et al., 1970). Little squalene is converted to cholesterol in liver (Sargent et al., 1970). The observation that chondrichthyans cannot make cholesterol from acetate (Diplock and Haslewood, 1965) implies the enzymes squalene monooxygenase and subsequent cyclases are absent. Teleost fish are reported to be able to synthesize cholesterol from mevalonic acid but this does not apply to chondrichthyans (Anon, 1983). It may be in those species that accumulate significant amounts of squalene, cholesterol synthesis is suppressed. Further studies of species with low squalene could confirm whether the entire group can synthesize cholesterol. The fact that squalene synthesis costs 18 ATP to synthesize from acetyl CoA and cannot be used as an energy source suggests there may be substantial other advantages to its accumulation that have yet to be established.

7.3. Lipid Transport

Elasmobranchs lack the non-esterified fatty acid (NEFA) binding protein albumin (Metcalf and Gemmell, 2005) as do some teleost fish (De Smet et al., 1998; Metcalf et al., 1999). Because agnathans have the protein (Gray and Doolittle, 1992; Doolittle, 1987; Metcalf et al., 2003) its absence in chondrichthyans must represent a loss that reflects the lack of

need for the protein. It has been suggested that the lack of albumin and its effect on the oncotic pressure of the blood may improve the rate of glomerular filtration (Ballantyne and Fraser, 2013). Another explanation is that fatty-acid binding to the hydrophobic site of albumin would be disrupted by urea making the protein useless for fatty-acid transport (Ballantyne, 1997). Most NEFA transport in elasmobranchs occurs via plasma lipoproteins, especially low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (Metcalf and Gemmell, 2005). Previous reports of albumin in elasmobranch plasma have failed to characterize protein sufficiently to conclude it is albumin. For example, albumin reported at 3 g per L has been reported in blue sharks, *Prionace glauca*, by an automated clinical assay (Moyes and Fragoso, 2006).

As would be expected in the absence of albumin, plasma NEFAs are generally several fold lower in elasmobranchs compared to teleosts (Speers-Roesch et al., 2008) but may range as high as 600 μM in captive *L. erinacea* (see Speers-Roesch and Treberg, 2010 for review). The high values are likely associated with plasma lipoproteins. Plasma NEFAs of *S. acanthias* are low (about 100 μM) and decline by 40% post feeding (Wood et al., 2010). Plasma NEFAs of tropical chondrichthyans have a lower n3/n6 ratio than temperate species (Speers-Roesch et al., 2008) similar to the situation in teleost species. PUFA levels range from 30–50 mol % of the fatty-acid pool (Speers-Roesch et al., 2008). Transport of lipids from the gut is mainly as chylomicrons, which make up one quarter of the plasma lipids in *S. acanthias* (Lauter et al., 1968). Plasma cholesterol levels are lower in elasmobranchs than in most teleost fish (Babin and Vernier, 1989; Larsson and Fange, 1977). LDL and high density lipoproteins (HDL) are involved in cholesterol transport as in most other vertebrates (Mills and Taylaur, 1971). Three fatty acid binding proteins (FABPs) resembling liver and heart types of mammals have been identified in liver of *Halaetunus bivias* (Cordoba et al., 1999). The liver type predominates and has a higher affinity for polyunsaturated fatty acids while the heart types have a greater specificity for saturated fatty acids (Cordoba et al., 1999).

7.4. Lipid Oxidation

The lack of albumin or a similar-sized fatty-acid transporting protein in chondrichthyans has been suggested to be responsible for the lack of significant extrahepatic lipid oxidation (Ballantyne, 1997). Carnitine palmitoyl transferase (CPT) is found in activities similar to those of liver in rectal gland and kidney (Speers-Roesch et al., 2006a), which indicates these tissues can use fatty acids as oxidative substrates. mRNA for 2 isoforms of CPT-1 have been reported in red muscle and heart of *S. acanthias* but it is

not yet known if a functional protein is expressed (Speers-Roesch and Treberg, 2010). It would appear that in tissues where ATP needs to be supplied at high rates, lipid oxidation is not an option due to the absence of albumin. Other tissues with lower ATP demands such as rectal gland and kidney may be able to use lipid that is delivered by a slower non-albumin related system as described above. Activation of liver CPT 1 has been observed in food deprived *S. acanthias* associated with an elevation of plasma BHB (Treberg et al., 2006a) indicating the capacity to upregulate lipid oxidation to provide ketone bodies to extrahepatic tissues.

7.5. Ketone Body Metabolism

In most vertebrates, ketone bodies are the only fuels that can replace glucose in some tissues such as the brain and their use is generally restricted to periods of starvation when glucose availability is reduced. Elasmobranchs differ from the vertebrate paradigm in that in nonstarving conditions ketone bodies seem to be important oxidative substrates. High β -hydroxybutyrate dehydrogenase (BHBDH) activities occur in liver, rectal gland, kidney, heart, and red muscle (Moon and Mommsen, 1987) suggesting most tissues use ketone bodies under normal fed conditions. Based on enzyme activities BHB is an especially important fuel for muscle (Watson and Dickson, 2001) and this has been confirmed in exercising *S. acanthias* (Richards et al., 2003). Similarly, the rectal gland has a high capacity to use ketone bodies (Walsh et al., 2006b; Speers-Roesch et al., 2006a). It has been suggested that 20% of the ATP needed for recovery from exhaustive exercise is provided by ketone body oxidation (Richards et al., 2003). Ketone bodies may play a role in hypoxia or anoxia as suggested for some teleosts (LeBlanc and Ballantyne, 2000). Plasma BHB concentrations decrease in hypoxia (Speers-Roesch et al., 2012a).

Plasma BHB levels range from about 1 mM in fed to 17 mM in fasted fish (see Speers-Roesch and Treberg, 2010, for summary of values across species). Plasma BHB levels rise 2–3 fold after 2 months fasting in *S. acanthias* (Wood et al., 2010) and *S. canicula* (Zammit and Newsholme, 1979). Acetoacetate concentrations range from 0.07 to 0.5 mM and do not change with physiological status to the same extent as BHB (see Speers-Roesch and Treberg, 2010 for summary of values). There are seasonal variations in ketone body levels in *Scyliorhinus canicula* (Gutierrez et al., 1988). Plasma BHB levels fall markedly after feeding but return to control values after 1–2 days (Wood et al., 2010).

Other pathways may contribute to ketone body synthesis. Ketogenesis from alanine has been demonstrated in the liver of *S. acanthias* (Anderson, 1990). One of the enzymes involved, hydroxymethylglutaryl CoA lyase,

has been reported in liver and kidney (Berges and Ballantyne, 1989) and acetyl CoA thiolase but not hydroxymethylglutaryl CoA synthase activity increases in liver of food deprived *S. acanthias* associated with elevated BHB levels (Treberg et al., 2006a).

Transport of ketone bodies across membranes occurs via a monocarboxylate transporter similar or identical to the one used to transport lactate. The monocarboxylate transporter MCT1 has been localized in the shark brain to the blood cerebrospinal fluid barrier (Balmaceda-Aguilera et al., 2012). MCT1 has also been localized to the endothelial cells of the brain suggesting its role in moving ketone bodies into the brain as in mammals (Balmaceda-Aguilera et al., 2012). This unusual since the blood brain barrier is between glial cells, not between endothelial cells.

7.6. Eicosanoid Metabolism

The eicosanoid system of chondrichthyans is somewhat simpler than that of the higher vertebrates (Fig. 7.9). It is based on arachidonic acid as in other vertebrates. Arachidonic acid is one of the main fatty acids in

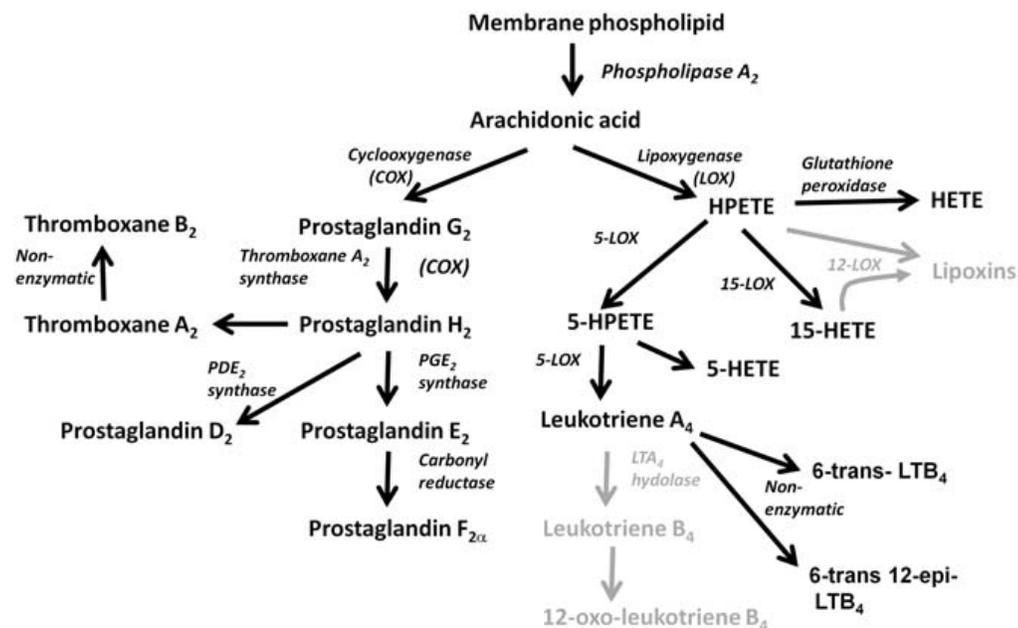


Figure 7.9. Eicosanoid pathways occurring in chondrichthyans. Enzymes and intermediates in black font are known to occur in chondrichthyans. Enzymes and intermediates in gray font do not occur in chondrichthyans. LTB₄, leukotriene B₄; HETE, hydroxyeicosatetraenoic acid; HPETE, 5-hydroperoxyeicosatetraenoic acid; PDE₂, prostaglandin E₂. Based on Ogata (1975), Pettitt and Rowley (1991), Rowley et al. (1987), Yang et al. (2002), Morgan et al. (2005), Thomson et al. (1998), and Cabrera et al. (2003).

phosphatidyl inositol from the electric organs of *Discopyge* and *Torpedo* at ~20% as in other vertebrates (Rotstein et al., 1987). Elasmobranchs have a single cyclooxygenase (sCOX = COX2) (Yang et al., 2002) that produces prostaglandins G₂ and H₂. Higher transcript levels of COX2 are found in hypoxia-adapted epaulette sharks (Rytkonen et al., 2010), which reinforces the role of prostaglandins rather than NO in mediating tissue perfusion of chondrichthyans.

The 5- and 15-lipoxygenase are present and 5-hydroperoxyeicosatetraenoic acid (5-HPETE), leukotriene A₄ (LTA₄), 6-trans-leukotriene B₄, 6-trans-12-epi-leukotriene B₄, 5(S),6(R) dihydroxyeicosatetraenoic acid, and 5- and 15-hydroxyeicosatetraenoic acids (5-HETE and 15-HETE respectively) are produced (Pettitt and Rowley, 1991). Several related enzymes are not present, including the 12-lipoxygenase (12LOX) (Fig. 7.9), and thus elasmobranchs cannot synthesize lipoxins (Pettitt and Rowley, 1991). The absence of 12LOX in elasmobranchs is interesting because in mammals the metabolites of this enzyme have been identified as procarcinogenic (Pidgeon et al., 2007). Elasmobranchs lack leukotriene A₄ hydrolase and thus cannot produce LTB₄ (Morgan et al., 2005; Pettitt and Rowley, 1991). LTB₄ is involved in the inflammatory response in higher vertebrates mediating the adhesion and activation of leucocytes to the endothelial cells of the capillaries. Its absence in chondrichthyans suggests a different mechanism for infiltration of leucocytes into the affected areas of a wound. Prostaglandins D₂, F_{2α}, and E₂ are produced in leucocytes of *S. acanthias* (Rowley et al., 1987). Prostaglandin E₂ has been found in intestine of *Triakis scyllia* (Ogata, 1975).

Prostaglandin E₁, produced from the n6 fatty acid dihomo-γ-linolenic acid, has not been detected in elasmobranchs (Ogata, 1975). The role of n3 series fatty acids in affecting or contributing to eicosanoid pathways is poorly understood. Prostaglandin E₃, an eicosanoid metabolite of eicosapentaenoic acid an n3 fatty acid has not been detected in elasmobranchs (Ogata, 1975).

Thromboxane B₂ (TXB₂) a metabolite of TXA₂, has been reported in serum (Thomson et al., 1998) and plasma (Cabrera et al., 2003) of several elasmobranchs but it is not known if it has a function. In mammals, it is excreted and has no known role.

8. VITAMIN METABOLISM

While the dietary vitamin requirements of several commercially important teleost fish species have been established (Anon, 1983), little is known of the vitamin requirements of elasmobranchs. In husbandry

situations teleost requirements are used as benchmarks for elasmobranchs (Janse et al., 2004). Much more work needs to be done in this area to optimize vitamin contents of diets for captive elasmobranchs. Elasmobranchs can synthesize ascorbate based on the presence of the gene for L-gulonolactone oxidase (Cho et al., 2007), and measurements of the enzyme activity in kidney (Fracalossi et al., 2001). This capacity is similar to the capacity of other primitive fish. Teleost fish lack the enzyme. Ascorbate levels in muscle are lower than those of teleost fish that lack the capacity for its synthesis (Velez-Alavez et al., 2014). Vitamin E levels are lower in elasmobranchs than in teleosts (Velez-Alavez et al., 2014) but vitamin D is found at high levels similar to those of teleost fish (Di Giulio and Hinton, 2008).

9. XENOBIOTIC METABOLISM

Compared to the vast literature for teleosts (Kennedy, 1995; Di Giulio and Hinton, 2008) little is known of the xenobiotic metabolism of elasmobranchs. The large amounts of lipid in the livers of elasmobranchs constitute a reservoir for the accumulation of lipid soluble toxicants. This coupled with their relative longevity and position as apex predators could make some species more susceptible to environmental pollution. A compilation of levels of many of the toxic organic compounds is found in (Gelsleichter and Walker, 2010). Chondrichthyans do have the metabolic capacity to detoxify and excrete toxicants although mixed function oxidase activity in liver of elasmobranchs is much lower than that of mammals (Bodine et al., 1985) and they lack nitro-reductase (Adamson et al., 1965). The potent carcinogen aflatoxin B₁ is detoxified to aflatoxicol in elasmobranch liver preparations via a nonmixed function oxidase pathway, while mammal liver converts it to aflatoxin Q₁ and M₁ via mixed function oxidase (Bodine et al., 1989). Four glutathione S-transferases differing in their stereospecificity have been isolated from liver of *L. erinacea* (Foureman et al., 1987), which indicates the capacity to detoxify a range of toxic compounds. Increased ethoxyresorufin-O-deethylase (EROD) activity and Cytochrome P450 1A protein content are inducible in elasmobranchs but not agnathans indicating the presence of the aryl hydrocarbon receptor (Hahn et al., 1998). Ethylmorphine demethylase and benzopyrene hydroxylase have been found in *S. acanthias* (Andersson and Nilsson, 1989). The rectal gland can excrete xenobiotics with the multidrug resistance protein MRP2, the only xenobiotic transporter found in that tissue (Miller et al.,

1998). Maternal transfer of organic toxicants to offspring in utero is thought to occur in white sharks *Carcharodon carcharias* (Mull et al., 2013).

The handling of heavy metals by elasmobranchs has been recently reviewed (Gelsleichter and Walker, 2010). Methylmercury is excreted slowly in the bile of *L. erinacea* and *S. acanthias* (Ballatori and Boyer, 1986) as would be expected considering the slow rate of bile formation as discussed above (see Section 3.4). Heavy metals can be handled by flavin-containing monooxygenases (Schlenk and Li-Schlenk, 1994) and by metallothioneins (Hidalgo et al., 1985) that have been found in some species. Elasmobranchs are 10-fold more sensitive to the toxic effects of silver compared to marine teleosts (De Boeck et al., 2001). Copper is toxic to elasmobranchs at levels slightly lower than that for marine teleosts and acts by compromising the gill permeability to urea with concomitant urea losses (De Boeck et al., 2007).

10. CONCLUSIONS AND PERSPECTIVES

An examination of the differences between chondrichthyan metabolism and that of other vertebrates can benefit the husbandry of the group as well as promote the understanding of the evolution of metabolism in the vertebrates. From a mammalian perspective chondrichthyans seem primitive anatomically, physiologically, and biochemically but we should keep in mind that the chondrichthyan fishes are the earliest known vertebrates to do many things. When compared to the Agnathans they show many important metabolic improvements. Many of these are summarized in Table 7.1. Anatomically, the development of jaws improved food acquisition and with some innovations in digestion including a stomach with acid secretion and some new digestive enzymes, digestion could occur at a faster (perhaps more efficient) rate. Further innovations in developing tissue specific isoforms improved tissue function but many of the isoforms found in higher animals required a genome duplication that did not involve the elasmobranchs. Fewer enzyme isoforms are found in glycolysis, nitric oxide metabolism, and creatine kinases. Along with the metabolic simplicity of the group, there is a regulatory simplicity (simpler eicosanoid pathways) and several examples where the multimeric enzyme complexes found in higher vertebrates are absent (e.g., cytochrome oxidase and the pyrimidine pathway enzymes). The lack of cholesterol synthesis (if true) is unique in the vertebrates. These examples inform us that a simple pattern of metabolic organization can serve as well as the more complex patterns found in the teleosts and higher vertebrates. Current threats aside, this group has weathered the major extinctions of the past as well or better than many other animal groups.

The importance of urea synthesis to the overall organization of chondrichthyan metabolism cannot be over-stated. Any pathways needing glutamine (e.g., pyrimidine synthesis) are suppressed in liver and mechanisms put in place to help stabilize plasma glutamine and conserve nitrogen from loss by excretion.

Within the chondrichthyans there are diverse patterns for many features. For example, glucose uptake in the kidney differs between sharks and skates, and there is a single gene for glutamine synthetase in elasmobranchs but two in holocephalans. Differences also exist in the response of the urea synthesis pathway to salinity stress.

Although the chondrichthyans are not in the main line of vertebrate evolution, it is tempting to speculate that their metabolism is similar to the metabolic organization of the ancestral jawed vertebrate. The fact that the coelacanth, from a vertebrate lineage almost as old as that of the chondrichthyans, are also urea-retaining, suggests the ancestral jawed vertebrate was a urea-retaining marine animal.

It is hoped this review will help generate greater interest in the chondrichthyans from a metabolic perspective. There are still many areas needing investigation. Membrane transporters including the monocarboxylate carrier amino acid transporters, especially those for glutamate, glutamine and arginine, should be characterized physiologically to better understand the regulation of nitrogen metabolism. A more complete characterization of chondrichthyan eicosanoid and n3 and n6 fatty acid metabolism would benefit understanding many other areas of their physiology including membrane lipid dynamics and their response to disease and stress. A better comprehension of the vitamin requirements of the group would benefit the husbandry of captive chondrichthyans.

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ENDOCRINE SYSTEMS IN ELASMOBRANCHS

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Elasmobranchs present numerous challenges as we uncover the evolutionary history of endocrine systems in vertebrates. Urea retention, metabolism, renal physiology, cardiorespiratory function, rectal gland function, and the many varied reproductive strategies are just some of the unusual and sometimes unique aspects of elasmobranch physiology. These differences in physiology can lead to significant challenges in interpretation of endocrine data from elasmobranch fish. Taking a classical endocrine approach this chapter focusses on the source of key endocrine systems, stimulus for release, and action at the target site of predominantly blood-borne hormones. A comprehensive coverage of pituitary hormones highlights the limited knowledge we have on the diverse role these hormones play in regulating whole-body physiology in elasmobranchs. Examination of interrenal endocrinology discusses the unique nature of 1α -hydroxycorticosteroid and the role of humorally derived catecholamines on cardiorespiratory

function. Gastro-entero-pancreatic (GEP) hormones in elasmobranchs have been reasonably well characterized but functional studies have, with the exception of the rectal gland, focused on the vasoactive nature of these hormones. Thus we have limited knowledge on the endocrine control of solute transport and energy balance by GEP hormones in elasmobranchs. Unlike teleosts and mammals elasmobranchs have a single natriuretic peptide – CNP released from the heart. The role of CNP is discussed in the context of salt and water balance and integrated with the bioactive component of the renin angiotensin system, angiotensin II. This chapter closes with a current understanding of hormones involved in calcium regulation in elasmobranchs. Comparisons between species are made throughout this chapter, which frequently results in an equivocal understanding of the role of hormones in regulating key physiological function underscoring the diverse nature of this ancient group of fishes.

1. INTRODUCTION

In 1903, as part of a part of a series of publications, William Bayliss and Ernest Starling published a paper that many consider as the birth of comparative endocrinology ([Bayliss and Starling, 1903](#)). Bayliss and Starling were interested in what stimulated the exocrine activity of the pancreas in mammals. Using the dog as a model they had demonstrated that this was not a reflex response, akin to contemporary experiments conducted by Pavlov on dogs. Rather a blood-borne agent or hormone, (from the Greek ‘I excite or arouse’) as Starling was later to describe it in his address to the Royal College of Physicians in London, was being released from the anterior intestine into the circulation. The paper demonstrated that intravenous administration of acid extracts from the anterior intestine of a dogfish into the dog, stimulated exocrine activity from the pancreas of the dog. The hormone was named secretin and it is now recognized as one of the hormones in the secretin super-family, with one of its primary functions to stimulate the release of pancreatic juice into the duodenum in response to the introduction of acidic chyme from the stomach. In choosing the dogfish, Bayliss and Starling demonstrated the ancient evolutionary origin of a secretin-like substance in vertebrates, and elasmobranchs remain a pivotal model in investigations of the evolutionary origins of vertebrate endocrine systems. Historical studies used mammalian-based antibodies to provide support for the presence of hormones and hormonal systems in elasmobranchs, with contemporary studies using molecular techniques to support or refute these findings. Interestingly, despite the pioneering work of Bayliss

and Starling over 100 years ago, secretin remains to be characterized in the intestine of elasmobranchs. Using the classic endocrine paradigm of source, stimulus, and target, this chapter will focus primarily on blood-borne hormones that have been identified in elasmobranchs and when known, discuss the role these hormones are thought to play in regulation of physiological systems.

2. PITUITARY GLAND

The fish pituitary gland is composed of both an adenohypophysis and a neurohypophysis. While there are considerable morphological nuances (for review see [Batten and Ingleton, 1987](#); [Cerdá-Reverter and Canosa, 2009](#)) the adenohypophysis can be broadly categorized into three distinct regions ([Fig. 8.1](#)); the rostral pars distalis (RPD), proximal pars distalis (PPD), and the pars intermedia (PI). In elasmobranchs there is an extension from the RPD, the ventral lobe of the pars distalis (VPD). The VPD is often encased in cartilage and connected to the RPD by a thin connecting stalk ([Batten and Ingleton, 1987](#)). The neurohypophysis extends with finger-like projections into the PI with the two structures being intimately entwined. This close association often leads them to be referred to collectively as the neurointermediate lobe (NIL) ([Cerdá-Reverter and Canosa, 2009](#)). There is also additional evidence to suggest some neurohypophysial projections into the RPD ([Meurling et al., 1996](#)).

In fish the classic adenohypophysial and neurohypophysial hormones are all known to be present and secreted from specialized cells within the various regions of the pituitary ([Cerdá-Reverter and Canosa, 2009](#); [Fig. 8.1](#)). The adenohypophysis has three distinct families of peptide hormones: the growth hormone (GH) family including GH, prolactin (PRL), somatolactin (SL) and placental lactogen (PL) with evidence to support the presence of both GH and PRL in chondrichthyans ([Kawauchi and Sower, 2006](#); [Moriyama et al., 2008](#); [Huang et al., 2009](#); [Roch et al., 2011](#)). The glycoprotein family of hormones is heterodimeric glycoproteins with a conserved α subunit within a given species and a hormone specific β subunit. This family includes thyroid stimulating hormone (TSH) and gonadotrophic hormones GTH I and GTH II, which are functionally analogous to the mammalian FSH and LH respectively. β subunits orthologous to FSH and LH β subunits in actinopterygians have been identified in the European Lesser Spotted dogfish *Scyliorhinus canicula* ([Quérat et al., 2001](#)); however, while thyrotropin activity has been reported from the ventral lobe of the elasmobranch pituitary ([Dent and Dodd, 1961](#)) molecular identification of a

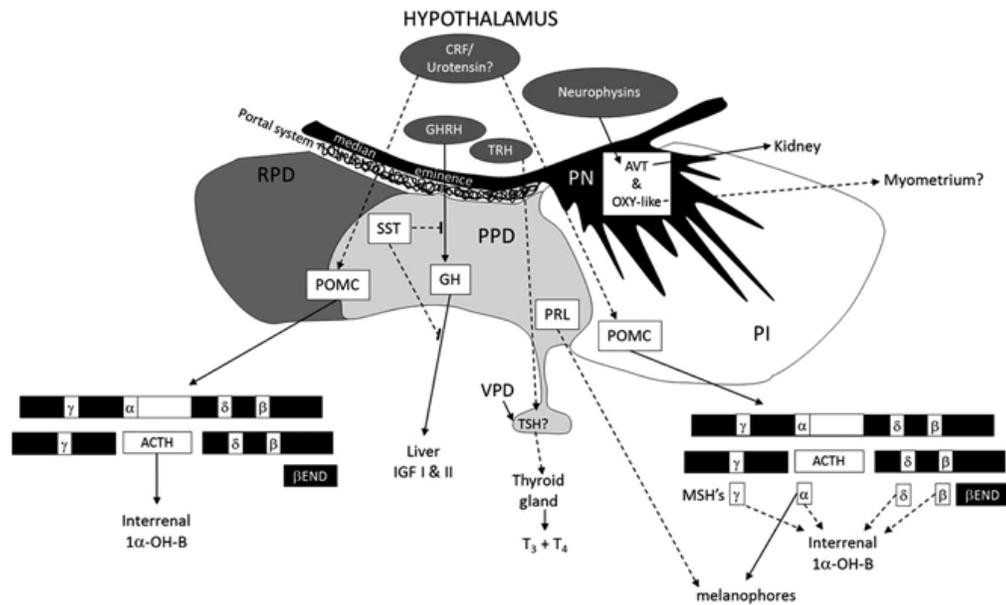


Figure 8.1. Basic anatomy of the elasmobranch pituitary and the hormones released. The Rostral Pars Distalis (RPD), Proximal Pars Distalis (PPD) and Ventral Lobe of the Pars Distalis (VPD) make up the anterior pituitary or the adenohypophysis. The Pars Intermedia (PI) with the Pars Nervosa (PN) collectively form the Neuro-intermediate Lobe (NIL). Solid arrows represent confirmed hormone release and target, although it is not clear if some hypothalamic factors are first released into the portal blood supply or synapse directly with pituitary cells. Dotted arrows represent presumed hormone release and target. Hormones with a question mark represent uncertain source/identification in elasmobranchs. 1α -OH-B, 1α -hydroxycorticosterone; ACTH, adrenocorticotrophic hormone; AVT, arginine vasotocin; β END, β endorphin; CRF, corticotrophic releasing factor; GHRH, growth hormone releasing hormone; IGF I & II, insulin-like growth factors I & II; MSH $\alpha, \beta, \delta, \gamma$, melanocyte stimulating hormones; OXY-like, the neutral neurohypophysial peptides; POMC, pro-opiomelanocortin; PRL, prolactin; SST, somatostatin (possible inhibition); TRH, thyrotrophic releasing hormone; TSH, thyroid stimulating hormone. See text for physiological details. Figure adapted from [Liang et al. \(2013\)](#) and [Batten and Ingleton \(1987\)](#).

β TSH subunit in elasmobranchs remains elusive ([Roch et al., 2011](#)). The final family of adenohypophysial hormones is derived from the precursor peptide pro-opiomelanocortin (POMC). Posttranslational modification of this precursor peptide can be significant and is largely dependent on the expression of proconvertase enzymes, which are tissue specific. In most vertebrates there are three principle regions; the N-terminus, which contains pro- γ -melanocyte-stimulating hormone (MSH); a central region, which contains the peptide sequence for adrenocorticotrophic hormone (ACTH), with α -MSH at the N-terminus of ACTH; and the C-terminus region, which contains β -lipoprotein and β -MSH is nestled within this peptide ([Kawauchi and Sower, 2006](#)). Elasmobranchs contain a fourth MSH, that is δ -MSH

(Amemiya et al., 1999, 2000) located between the α and β forms of MSH and thought to be the result of a gene duplication and subsequent mutation of the gene encoding the β -MSH and β endorphin (β END) portion of the precursor peptide (Amemiya et al., 1999, 2000; Fig. 8.1).

The neurohypophysis contains two types of hormones that are released on demand into the circulation in response to the relevant stimuli. Both types of hormone are nonapeptides (nine amino acids) and include the neutral or oxytocin-like hormones and the basic or vasopressin-like hormones.

2.1. Growth Hormone Family

2.1.1. GROWTH HORMONE — IGF AXIS

GH release from the mammalian pituitary is under the control of growth hormone releasing hormone (GHRH) (stimulatory) and somatostatin (SST) (inhibitory). Definitive evidence supporting GHRH presence in the brain of elasmobranchs is thus far limited to a single study on the spiny dogfish, *Squalus acanthias* (Plesch et al., 2000); however, somatostatin has been identified by biochemical and molecular means in *S. canicula* and the Electric Ray, *Torpedo marmorata* (Conlon et al., 1985; Quan et al., 2013). A growth hormone-like protein first identified from the pars distalis region of the Blue Shark, *Prionace glauca* (Lewis et al., 1972) was later sequenced (Hayashida and Lewis, 1978; Yamaguchi et al., 1989) and the gene subsequently identified from the PPD of *S. acanthias* (Moriyama et al., 2008). *S. acanthias* shares 94% identity with *P. glauca* GH sequence and between 37–66% with GHs identified in telesots (Moriyama et al., 2008).

In all vertebrates thus far examined, growth hormone released from the pituitary will typically target the liver to stimulate synthesis and release of insulin-like growth factors I and II (IGF-I and II), which in turn stimulate growth of a variety of tissues. This is known as the somatomedin hypothesis and growth of cartilage in the mammalian epiphyseal plate has been used as a classic assay to demonstrate the dual nature of GH and IGF-I control in long bone growth (Le Roith et al., 2001). The presence of IGFs in elasmobranchs was first indicated using a heterologous assay where the skeleton of a shark was homogenized and subsequent measurement of both IGF-I and II was conducted using a heterologous mammalian based assay (Bautista et al., 1990). More concrete evidence was obtained from IGF-I-like immunoreactivity in the intestinal mucosa and plasma of the Thornback Ray, *Raja clavata* (Reinecke et al., 1992) and ligand-binding studies showing IGF-I binding in the brain and liver of *T. marmorata* (Drackenberg et al., 1993). IGF-I-like immunoreactivity in somatostatin and glucagon secreting

cells and IGF-II-like immunoreactivity in insulin secreting cells of the pancreas in *R. clavata* has also been demonstrated (Reinecke et al., 1994) suggesting communication between and within pancreatic islet cells. Definitive molecular identification of the presence of both IGFs in elasmobranchs was provided from liver tissue of *S. acanthias* (Duguay et al., 1995) where it was shown that IGF I and II share at least 60% homology with human IGF I and II respectively.

Homologous GH has been shown to stimulate both IGF I and II transcription from the elasmobranch liver *in vitro* (Moriyama et al., 2008) and further evidence to support the somatomedin hypothesis in elasmobranchs is implied in the original identification of IGF in elasmobranchs where purified shark GH significantly stimulated growth in the rat tibia assay (Hayashida and Lewis, 1978). More direct evidence was provided in the Clearnose Skate, *Raja eglanteria* (Gelsleichter and Musick, 1999), when recombinant human IGF was shown to enhance sulfate uptake in vertebrae, a clear indication of growth regulation in cartilage. Furthermore, the addition of human IGF-I has been shown to stimulate cartilage growth in culture in the Ocellate Spot Skate, *Raja porosa* (Fan et al., 2003). An additional role in regulating reproductive capacity in elasmobranchs was demonstrated where human IGF-I was shown to enhance DNA synthesis through uptake of ³H thymidine in cultured spermatocytes of *S. acanthias* (Dubois and Callard, 1993).

2.1.2. PROLACTIN

There are no reports on the molecular identification of prolactin (PRL) in elasmobranchs. The closest molecular evidence comes from the identification of a related peptide, prolactin 2 (PRL-2) in a holocephalan, the Elephantfish, *Callorhinchus milii* (Huang et al., 2009) with PRL-1 first appearing in teleosts (Roch et al., 2011). Thus, evidence to support the existence and functional role of PRL in elasmobranchs is largely circumstantial and based on physiological studies using heterologous PRL. In vertebrates, PRL is known for its diverse forms and subsequent physiological actions on reproduction, osmoregulation, growth and development, pigmentation, and molting. There is an implication that a PRL-like hormone may be involved in the control of osmoregulation in elasmobranchs, as following transfer from 100% to 33% seawater, there was a 100-fold increase in pituitary PRL-like activity in the Atlantic Stingray, *Dasyatis sabina* (De Vlaming et al., 1975). PRL may also be involved in regulating color and luminescence in elasmobranchs (Visconti et al., 1999; Claes et al., 2012). In a luminescent shark, *Squaliolus aliae*, the regulatory role of PRL appears indirect through stimulation of pigment expansion in melanophores that cover the photogenic cells regulating the amount of light produced

(Claes et al., 2011). Similarly, dispersal of pigment in melanophores following human PRL administration was observed in Smooth Backed River Stingray, *Potamotrygon reticulatus*, where it was shown that the actions of PRL on the melanophores was likely via the cAMP signaling pathway (Visconti et al., 1999). There are no reports of the presence or physiological activity of the final two members of the growth hormone family of peptides, somatolactin and placental lactogen, or variants thereof, in elasmobranchs.

2.2. Glycoprotein Family

Regulation of sex steroid synthesis and reproduction in elasmobranchs is, as with all other vertebrates, highly dependent on the expression and actions of α and β subunits of FSH and LH or the equivalent pituitary hormones. Both have been identified in elasmobranchs with research effort on the function of these hormones naturally focusing on their role in reproduction. As a consequence FSH and LH will be discussed in detail in Chapter 7, Vol. 34A and here we will focus on the thyroid hormone system.

2.2.1. THYROID HORMONES

The first step in the thyroid hormone system pathway is the release of thyroid releasing hormone (TRH) from hypothalamic centers. TRH will then target thyrotropes in the adenohypophysis to initiate release of TSH (Fig. 8.1), which then targets the thyroid gland to stimulate synthesis and release of thyroid hormones. Thyroid releasing hormone-like immunoreactive nerve fibers have been identified in the neuroepithelium of the neurohypophysial stalk and to a lesser extent in the NIL of *S. canicula* (Tejido et al., 2002) and evidence of similar immunoreactivity in the ventral lobe of *S. acanthias* has been reported (Meurling and Rodríguez, 1990). The β subunit of TSH first appears in the teleost lineage (Roch et al., 2011); however, it is clear that elasmobranchs possess a thyroid endocrine axis analogous to other vertebrates. Incubation of the thyroid gland from *D. sabina* with pituitary homogenates from *D. sabina* significantly increased thyroid hormone production; however, incubation of the thyroid gland from *D. sabina* with mammalian TSH failed to elicit a response (Jackson and Sage, 1973), which suggests significant changes in the peptide structure of TSH and/or the TSH receptor between mammals and elasmobranchs. Furthermore, TSH-like activity was found to be located within the ventral lobe of the PPD in *S. canicula* (Dent and Dodd, 1961). With the lack of evidence for an elasmobranch TSH the question becomes what may stimulate thyroid gland activity in this group of fishes? One possible candidate is thyrostimulin; although not considered a classical pituitary

hormone it exists as a heterodimer with an α and β subunit and is considered the evolutionary precursor to FSH, LH, and TSH (Roch et al., 2011).

The thyroid gland in elasmobranchs, unlike the diffuse thyroid gland in teleosts, is encapsulated as a single collection of thyroid follicles and is arranged as a discrete structure centered ventrally to the pharynx close to where the ventral aorta bifurcates to supply the first and second branchial arteries. It is arranged in the typical follicular formation with a colloid space surrounded by follicular cells (Ferguson, 1911). Morphological studies of follicular cell height, nucleus height, and colloid area are frequently used to assess thyroid growth and activity. These parameters allow one to relate thyroid gland activity and function to a number of factors including exposure to environmental contaminants as potential causes of goiter (Crow et al., 2001) or life stage (Volkoff et al., 1999) where seasonal changes in morphology are likely linked to reproductive state (Volkoff et al., 1999). The follicular cell of the thyroid gland synthesizes the precursor molecule, thyroglobulin (TG) (Suzuki et al., 1975). TG is then transported into the colloid space where iodination and oxidative coupling are catalyzed by the thyroid peroxidase and NADPH oxidase systems to form the iodinated tyrosine units, mono-iodothyronine or di-iodothyronine. These molecules are the precursors to the two principle products of the thyroid gland, tetraiodothyronine (T_4) and triiodo-L-thyronine (T_3). These hormones are lipophilic; therefore, the vast majority of thyroid hormone in the circulation is bound to a carrier protein with a small fraction existing in the free form. Such carrier proteins have been identified in teleosts (Santos et al., 2002) but are currently unknown in elasmobranchs. The binding of thyroid hormones to plasma proteins means that only the free form is available for use at the target site and in teleosts only about 0.5 to 3.4% of the total circulating thyroid hormone exists in the free form (Eales and Shostak, 1987).

Thyroid hormones are involved in the reproductive cycle of vertebrates where their role appears to be largely permissive for other hormones involved in the regulation of metabolism and energy balance during reproduction and development (Karsch et al., 1995). Thyroidectomy impaired follicular development in *S. canicula* (Lewis and Dodd, 1974), and while circulating levels of free T_3 and T_4 in some species do not appear to be different between males and females (Lewis and Dodd, 1976; Crow et al., 1999; Volkoff et al., 1999), there is a distinct seasonal variation (Lewis and Dodd, 1976; McComb et al., 2005) that is most likely related to reproductive state (Volkoff et al., 1999). Thus, it would be informative to understand the carrying capacity (i.e., ratio of bound and free thyroid hormone in the circulation) of thyroid hormones in elasmobranch circulation to better explain potential seasonal changes associated with key life stages between sexes, such as early development or reproduction. Indeed

Volkoff et al. (1999) suggest that increases in T_4 and T_3 in mature females may be masked by increases in plasma vitellogenin that influence binding and carrying capacity of thyroid hormones. In the absence of binding studies this is entirely speculative at this stage.

In elasmobranchs, T_4 is typically the dominant form in the circulation (Suzuki et al., 1975) and T_3 is the principle bioactive form. Consequently there is significant intracellular regulation of the thyroid hormones in the form of deiodinases that selectively remove iodide from the outer or inner ring of the parent molecule to activate or deactivate T_4 respectively. There are three known deiodinases in vertebrates: D1, D2, and D3. D1 and D2 deiodinate the outer ring converting T_4 to the active T_3 . D1 and D3 deiodinate the inner ring and thus deactivate T_4 to rT_3 or additional inactive thyroid hormones (Leary et al., 1999; Martinez et al., 2008). Expression of deiodinases is species, tissue, and life-stage dependent, which highlights the crucial role these enzymes play in the fine tuning of thyroid function (Eales, 2006).

A T_4 outer ring deiodinase (ORD) has been partially identified in liver tissue from *S. acanthias* (Leary et al., 1999) and a corresponding inner ring deiodinase (IRD) analogous to D3 has been identified in the Brown Banded Shark, *Chiloscyllium punctatum* (Martinez et al., 2008). Both enzymes reportedly perform at similar rates with their teleost counter-part and both require the presence of organic osmolytes (urea and TMAO) in the incubation medium, albeit in different proportions, for optimum activity (Leary et al., 1999; Martinez et al., 2008). The authors argue that the gorge feeding behavior of many elasmobranchs, coupled with the plentiful supply of iodine in the marine environment may require a sensitive deactivating thyroid enzyme during times when uptake of thyroid hormone after a feeding event may be particularly acute. In support of this hypothesis there is a distinct relationship between diet and IRD activity in parasitic lamprey as T_4 and T_3 IRD is significantly higher in the intestine compared to the liver during the parasitic phase of their life cycle (Eales et al., 1997), thus regulating the uptake of active thyroid hormone from a blood meal.

It is well recognized that thyroid hormones are critical for appropriate development in all vertebrates. In mammals, maternal transfer of thyroid hormones across the placenta is essential for normal development (Calvo et al., 1992) and yolk concentration of thyroid hormone in teleosts influences developmental trajectory (Leatherland et al., 1989). Levels of T_3 and T_4 were measured in the maternal plasma and yolk of the placental viviparous shark, the Bonnethead Shark, *Sphyrna tiburo*, and both hormones were found to be consistently higher in the yolk compared to maternal plasma (McComb et al., 2005). Furthermore, there was a steady rise in yolk T_3 throughout development and one suggestion was an increase

in T_4 deiodination activity, which is supported by the measureable values of T_4 ORD in the yolk of *S. acanthias* (Leary et al., 1999). Presence of T_4 ORD in the yolk of the developing elasmobranch is unusual and does not agree with the teleost literature; however, it is important to note that the many varied reproductive strategies of elasmobranchs (see Awruch, Chapter 7, Vol. 34A) make it difficult to draw comparisons with teleosts in regard to maternal contribution of hormones to developing embryos.

$TR\alpha$ and β receptors bind T_3 at the target site and these have been identified by molecular means in teleosts (Yamano and Miwa, 1998) but are as yet unidentified in elasmobranchs. It is evident therefore that from activation to effect at the target site there are layers of potential regulation of the thyroid hormone system. However, the lack of information regarding regulation of the thyroid system in elasmobranchs was most clearly depicted by (Leary et al., 1999) where of the fifteen variables identified in thyroid function only four had been examined prior to 1999 and only a further three components have been examined since.

2.3. POMC Hormones

POMC acts as a major precursor to a number of peptides that can be broadly categorized into melanocortins and endorphins (Fig. 8.1). The melanocortins include melanocyte stimulating hormones (MSH), β -lipotropic hormone (β -LPH), and adrenocorticotrophic hormone (ACTH); and the endorphins include β -END and met-enkephalin. The corticotropes, found predominantly in the RPD of the pituitary in fishes including elasmobranchs (Batten and Ingleton, 1987; Takahashi et al., 2008), synthesize ACTH and β -LPH, whereas the melanotropes are predominantly located in the PI and mainly produce the MSHs (Lowry and Chadwick, 1970; Takahashi et al., 2008; Volkoff et al., 2009). Cellular dependent processing of POMC is achieved by the site specific expression of prohormone proconvertase (PC) enzymes (Tanaka, 2003) with PC-1 being responsible for the cleavage of ACTH and β -LPH from POMC in corticotropes and PC-1 and 2 being required for the production of MSHs in melanotropes. While there are a number of potential endocrine end products from POMC cleavage, only ACTH and MSH have been investigated in any detail in elasmobranchs.

2.3.1. ADRENOCORTICOTROPHIC HORMONE — ACTH

ACTH is a key component of the hypothalamo-pituitary-interrenal axis in fish, also known as the endocrine stress axis. The classic stress response involves corticotropic releasing factor (CRF) from the hypothalamus targeting corticotropes in the pituitary to stimulate synthesis and release

of ACTH. ACTH then targets melanocortin receptors (MCRs) on the interrenal cell membrane to stimulate corticosteroidogenesis and the release of the native steroid that ultimately leads to a mobilization of energy, usually in the form of glucose, which fuels restoration to the steady state (Wendelaar Bonga, 1997). In elasmobranchs this axis has not been fully characterized. For example CRF has yet to be identified in any elasmobranch species, but CRF belongs to a family of well-conserved peptides that includes the neuropeptides urotensin I (UI) and urotensin II (UII) (Lovejoy and Balment, 1999). A major source of urotensins in fish is the caudal neurosecretory region; a unique neuroendocrine structure in the distal region of the spinal cord of fishes that contains large peptidergic neurons, Dahlgren cells, that synapse with a neurohaemal area. In elasmobranchs the area is more diffuse than what has been described in teleosts but Dahlgren cells have been identified (Takei and Balment, 2009). Using an antibody raised against human 1–20 CRF that also cross reacted with urotensin I, CRF-like neurons were identified in the caudal spinal region of the Banded Houndshark, *Triakis scyllia* (Owada et al., 1985) and UI/CRF-like neurons were identified in the caudal neurosecretory region of the Big Skate, *Raja binoculata*, *S. acanthias*, and *D. sabina* (Onstott and Elde, 1986).

Both UI and UII have been isolated and characterized from the caudal neurosecretory system of *S. canicula* (Conlon et al., 1992b; Waugh et al., 1995) and UII has also been isolated from the brain of the Longnose Skate, *Raja rhina* (Waugh and Conlon, 1993). When administered *in vivo* to *S. acanthias*, UII induced a sustained hypertensive response (Hazon et al., 1993). In teleosts the stimulatory actions of UI on ACTH release have been described (Volkoff et al., 2009) and ACTH is present in the pituitary of *S. acanthias* (Lowry et al., 1974; Vallarino and Ottonello, 1987), *D. akajei* (Amemiya et al., 2000), *T. scyllia* (Takahashi et al., 2008), and *S. canicula* (Vallarino and Ottonello, 1987). Further indirect evidence for the presence of CRF or a CRF-like substance and the potential corticotrophic action in elasmobranchs was provided when the injection of human CRF into *S. canicula* led to an increase in plasma concentration of 1α hydroxycorticosterone (1α -OH-B), the principle corticosteroid in elasmobranchs (Conlon, J. M., Armour, K., and Hazon, N., unpublished; see Waugh and Conlon, 1993).

ACTH or MSH binds to the melanocortin receptors (MCRs). This is a family of G-protein coupled receptors with five MCRs thus far cloned in tetrapods (MC1R, MC2R, MC3R, MC4R, and MC5R) (Vastermark and Schiöth, 2011), six have been identified from the Zebrafish (*Danio rerio*) genome with the duplication of the MC5R gene, but only four have been identified on the Japanese Pufferfish genome (*Takifugu rubripes*) with

MC3R lost and only a single copy of MC5R expressed (Logan et al., 2003). In elasmobranchs only three – MC3R, MC4R, and MC5R – have thus far been identified (Ringholm et al., 2003; Klovins et al., 2004; Liang et al., 2013). Binding and functional expression studies have consistently shown a greater selectivity and potency for ACTH, regardless of the MCR being examined, and the binding affinity and potency for the MSHs to MCRs varies between studies (Liang et al., 2013). Interestingly, MC2R specifically binds ACTH with the other MCRs binding the various forms of MSH (Schiöth et al., 2005). Further MC2R is abundantly expressed in the head kidney of teleosts, which is the main site of cortisol synthesis (Aluru and Vijayan, 2008; Aguilero et al., 2010) and ACTH appears to be the predominant stimulus for corticosteroidogenesis from the teleost head kidney (Metz et al., 2005). Liang et al. (2013) suggested that while ACTH appeared to be the preferred and most active ligand for all three elasmobranch MCRs, binding affinity and functional expression studies using α , β , γ or δ MSH (Ringholm et al., 2003; Klovins et al., 2004; Haitina et al., 2007; Reinick et al., 2012) provide evidence for the potential of a dual regulatory route for the stimulation of corticosteroidogenesis, where RPD-derived ACTH and NIL-derived MSH (Takahashi et al., 2008) serve as the two possible stimuli.

2.3.2. MELANOCYTE STIMULATING HORMONE — MSH

In teleosts MSHs have been implicated in a number of different physiological responses, almost to a pleiotropic level with potential regulatory roles including, but not limited to, color change, food intake, lipid metabolism, and T_4 release from the thyroid gland (Volkoff et al., 2009). Expression of MC3R and MC4R in the brain (Liang et al., 2013) alongside identification of α -MSH in the hypothalamic feeding area in the brain of elasmobranchs (Demski, 2012) provides indirect evidence that α -MSH is involved in the regulation of food intake in elasmobranchs. A physiological role for MSH in elasmobranchs regulating color change has been reported with earlier studies showing hypophysectomy resulting in a distinct pallor in dogfish (Lundstrom and Bard, 1932; Parker, 1936). Subsequent research identified MSH as a major regulator of color change in elasmobranchs (Wilson and Dodd, 1973b). α , β , and γ MSH all induced a darkening of the skin in *S. canicula* with equal potency by dispersing melanin pigment in the melanophores on the skin (Sumpter et al., 1984). However, in the same study mammalian acetylated α MSH and synthetically acetylated dogfish α MSH were orders of magnitude more potent than the non-acetylated forms of MSH (Sumpter et al., 1984). This is an interesting finding as unlike mammals and teleosts acetylated forms of α MSH have not been identified in the PI of elasmobranchs (Lowry and

Chadwick, 1970; Takahashi et al., 2008) or indeed from whole pituitary extracts (Lorenz et al., 1986; Takahashi et al., 1999). It may be that post-translational modification of MSH occurs on or following secretion of MSH from the melanotropes in the PI.

2.4. Neurohypophysial Hormones

Two types of neurohypophysial peptides have been identified in all vertebrates to date, both contain nine amino acids with amino acids at positions 1, 5, 6, 7, and 9 being well conserved throughout the vertebrate phyla (Acher, 1996; Table 8.1). Arginine vasopressin (AVP) and the nonmammalian homologue, arginine vasotocin (AVT), both contain a basic residue (lysine or arginine) at position 8. Oxytocin-like peptides, of which there are many more variants in elasmobranchs, are known as the neutral peptides and all have a neutral residue (leucine, isoleucine, valine, or glutamine) at position 8. Of the seven different neurohypophysial peptides identified in elasmobranchs definitive physiological function has been assigned to only AVT despite concentrations of this peptide being the lowest in neurohypophysial extracts (Acher, 1996; Acher et al., 1999). Both peptides are synthesized and packaged in nerve bodies of the hypothalamus with particular concentration in the pre-optic nuclei (Meurling et al., 1996). Further, both peptides are packaged as precursor molecules with a specific signal peptide sequence and associated neurophysin. Using antibodies raised against elasmobranch neurophysins, immunoreactive fibers were tracked from the pre-optic nucleus into the NIL. In addition, some fibers terminated at the median eminence and a small proportion extended into the RPD in *S.*

Table 8.1
Comparative amino acid structure of basic (vasopressin-like) and neutral (oxytocin-like) neurohypophysial peptides

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|--|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Oxytocin – mammals | Cys | Tyr | Ile | Gln | Asn | Cys | Pro | Leu | Gly |
| Glunitocin – rays | | | | Ser | | | | Gln | |
| Valitocin – <i>S. acanthias</i> | | | | Gln | | | | Val | |
| Aspargtocin – <i>S. acanthias</i> | | | | Asn | | | | Leu | |
| Phasitocin – <i>T. scyllia</i> | | | Phe | Asn | | | | Ile | |
| Asvatocin – <i>S. canicula</i> + <i>T. scyllia</i> | | | | Asn | | | | Val | |
| Phasvatocin – <i>S. canicula</i> | | | Phe | Asn | | | | Val | |
| Vasopressin – mammals | Cys | Tyr | Phe | Gln | Asn | Cys | Pro | Arg | Gly |
| Vasotocin – elasmobranchs | | | Ile | | | | | | |

Table adapted from Hyodo et al., 2004b.

acanthias and the PPD in the Velvet Belly Lantern Shark, *Etmopterus spinax* (Meurling et al., 1996). The elasmobranch portal blood supply has both an anterior and posterior portion with the anterior supplying the RPD and the posterior supplying the PPD (Meurling, 1967). Thus, anatomical evidence suggests that neurohypophysial hormones may have significant regulatory influence over the adenohypophysial hormones either through direct neural connection or a neurohaemal route via the portal blood supply (Meurling et al., 1996).

2.4.1. OXYTOCIN-LIKE HORMONES

There are two oxytocin-like peptides identified in each of the shark species thus far investigated and only a single, glutitocin, in the rays (Buchholz et al., 1995; Acher et al., 1999; Hyodo et al., 2004b). It was postulated that the ureosmotic nature of elasmobranchs may be one reason why a number of different oxytocin-like neurohypophysial peptides have evolved in this group of fishes (Acher et al., 1999); however, direct empirical evidence supporting a role for any neurohypophysial hormone in urea transport in elasmobranchs is lacking despite the pivotal role of AVP in regulating renal urea transport in mammals (Sands, 2003).

Amino acid sequence analysis of the oxytocin-like peptides from *T. scyllia* demonstrated precursor packaging of these peptides similar to the oxytocin peptides in tetrapods and lungfishes with the signal peptide; the oxytocin or oxytocin-like peptide; and an associated neurophysin sequence (Hyodo et al., 2004b). This was also found in an isotocin-like precursor peptide identified in the preoptic nucleus of the brain in *T. marmorata* (Buchholz et al., 1995). Few studies have examined the physiological actions of oxytocin-like peptides in elasmobranchs. Aspartocin, valitocin, and phasvatocin were all examined for a potential regulatory role in rectal gland secretion and were without effect (Stoff et al., 1979). Additional studies have used heterologous bio-assays with uterine strips from the rat (Sawyer et al., 1969) or NIL extracts on myometrium from *S. acanthias* (Sorbera and Callard, 1995) to understand potential physiological function of the oxytocin-like peptides. The results in both these studies suggest oxytocin-like activity in the elasmobranch neurohypophysis; however, the structural identity of the bioactive hormone was never determined.

2.4.2. ARGININE VASOTOCIN-LIKE HORMONES

As in tetrapods and lungfish the gene structure of AVT in elasmobranchs has a signal molecule followed by the nonapeptide AVT followed by a neurophysin and finally a copeptin molecule (Hyodo et al., 2004b). AVT is located in neurons originating in the pre-optic nuclei of the hypothalamus

(Vallarino et al., 1990) where it is transported to the neurohypophysis, the precursor is catalyzed, and AVT is released on demand. In all vertebrates thus far examined there are two types of stimuli for the release of AVT, cellular and extracellular dehydration, which leads to an increase in cellular osmolality or a decrease in extracellular fluid volume respectively. The result is the same in that there is a net loss of total body fluid volume, but cellular dehydration is by far the more potent stimulus for AVT release in vertebrates (Takei and Balment, 2009). Hypothalamic expression and circulating levels of AVT were measured in *T. scyllia* following transfer to the hyperosmotic environment of 130% seawater and the hypoosmotic environment 60% seawater (Hyodo et al., 2004b). Expression levels were significantly higher than control in the 130% acclimated fish two days after transfer and circulating levels were significantly higher than the 60% acclimated fish 2 days after transfer (Hyodo et al., 2004b). In the euryhaline bullshark, *Carcharhinus leucas*, stepwise transfer from freshwater to 75% seawater then 100% seawater saw an increase in circulating levels of AVT within 96 h in 75% seawater compared to control, however, within 72 h of acclimation to 100% seawater circulating levels of AVT were not significantly different from those in freshwater control fish (Anderson et al., 2006). These data combined suggest a more acute regulatory role for AVT in elasmobranchs in relation to body fluid volume regulation and are consistent with the acute antidiuretic role AVT plays in teleosts (Takei and Balment, 2009).

The antidiuretic role of AVT on vertebrate kidneys is particularly well documented, so much so that the mammalian homologue, AVP, is perhaps best known as antidiuretic hormone (ADH). In mammals, AVP targets collecting duct epithelial cells in the kidney to increase aquaporin-2 and UT transporter abundance (Sands, 2003). The net effect is an increase in water reabsorption from the lumen of the nephron to the blood thereby reducing urine flow rate, increasing body fluid volume, and ultimately restoring cellular hydration. In the elasmobranch kidney there is little evidence to support a similar role at the epithelial level for AVT in water and urea reabsorption. This despite the fact that the elasmobranch kidney is well known to be particularly efficient at urea reabsorption (Boylan, 1972; see also Chapter 5) and UT is abundantly expressed in the collecting duct of the kidney in *T. scyllia* (Hyodo et al., 2004a). Nonetheless AVT is potently antidiuretic in the kidney of elasmobranchs (Wells et al., 2002), but all the evidence thus far suggests that the antidiuretic action of AVT results from vascular constriction controlling glomerular filtration rate (GFR) and perhaps single nephron glomerular filtration rate (SNGFR). Vascular corrosion casting of the glomerulus in *S. canicula* showed approximately one third of all nephrons having a vascular bypass shunt from the glomerular

afferent arteriole to the peritubular network (Brown and Green, 1992) and AVT perfusion in the *in situ* perfused kidney of *S. canicula* resulted in a 60% reduction in the population of perfused and filtering nephrons (Wells et al., 2005) indicative of glomerular bypass. Despite the huge reduction in filtering nephrons UFR was still maintained and it was suggested that SNGFR may have increased to compensate. Regulation of SNGFR by adrenaline had previously been demonstrated in the perfused kidney of *S. canicula* (Brown and Green, 1987).

Interactions between AVT and the pineal gland in addition to the now well-recognized regulatory role of AVT in ACTH release have been documented in mammals and teleosts (Rivier and Vale, 1983; Takei and Balment, 2009). However, investigations on the physiological role of AVT in elasmobranchs have not extended beyond the vascular and renal actions described here, although it is reasonable to suggest that AVT may regulate adenohipophysial peptides such as ACTH given the anatomical projection of some neurohipophysial neurons in close association with the RPD (Meurling et al., 1996).

3. CORTICOSTEROIDS AND CATECHOLAMINES

3.1. Interrenal Corticosteroids

Elasmobranchs are the most basal of vertebrates where corticosteroidogenic tissue of the interrenal gland is anatomically separate from chromaffin and renal tissue. The interrenal gland has an opaque off-white yellow color and in sharks is arranged along the length of the kidneys as a string of tissue running centrally between each kidney with wider aggregations of steroidogenic cells at the posterior end of the kidney, or in some cases as several sections of rod-like structures arranged centrally along the length of the kidney. In skates and rays that have a more compact kidney, the interrenal gland is similarly more compact and again lies between the bilobed kidneys often in a U shape, again with denser aggregations at the posterior end (Butler, 1973; Chester-Jones and Phillips, 1986). Ultrastructural studies of the interrenal tissue in elasmobranchs highlight cells with numerous mitochondria with tubular shaped cristae that are a hallmark of steroidogenic cells: lipid droplets and smooth endoplasmic reticulum. It is not clear if the elasmobranch interrenal gland is zoned, as seen so strikingly in the mammalian adrenal gland; with the only evidence supporting zonation from ultrastructural studies of the interrenal gland in the nurse shark, *Ginglymostoma cirratum* (Taylor et al., 1975).

A second key characteristic of the elasmobranch interrenal gland is that it produces a corticosteroid unique to elasmobranchs, 1α -hydroxycorticosterone (1α -OH-B) (Idler and Truscott, 1966, 1967; Truscott and Idler, 1968, 1972). The homogeneous nature of the interrenal gland alongside the single corticosteroid produced from the gland make a compelling case for the interrenal gland of elasmobranchs as a model for the study of corticosteroidogenesis in vertebrates. However, one factor that has severely hampered the study of corticosteroidogenesis in elasmobranchs has been our inability to elucidate the complete biosynthetic pathway (Fig. 8.2).

The first step in the steroidogenic pathway requires the translocation of cholesterol from the outer mitochondrial membrane to the inner

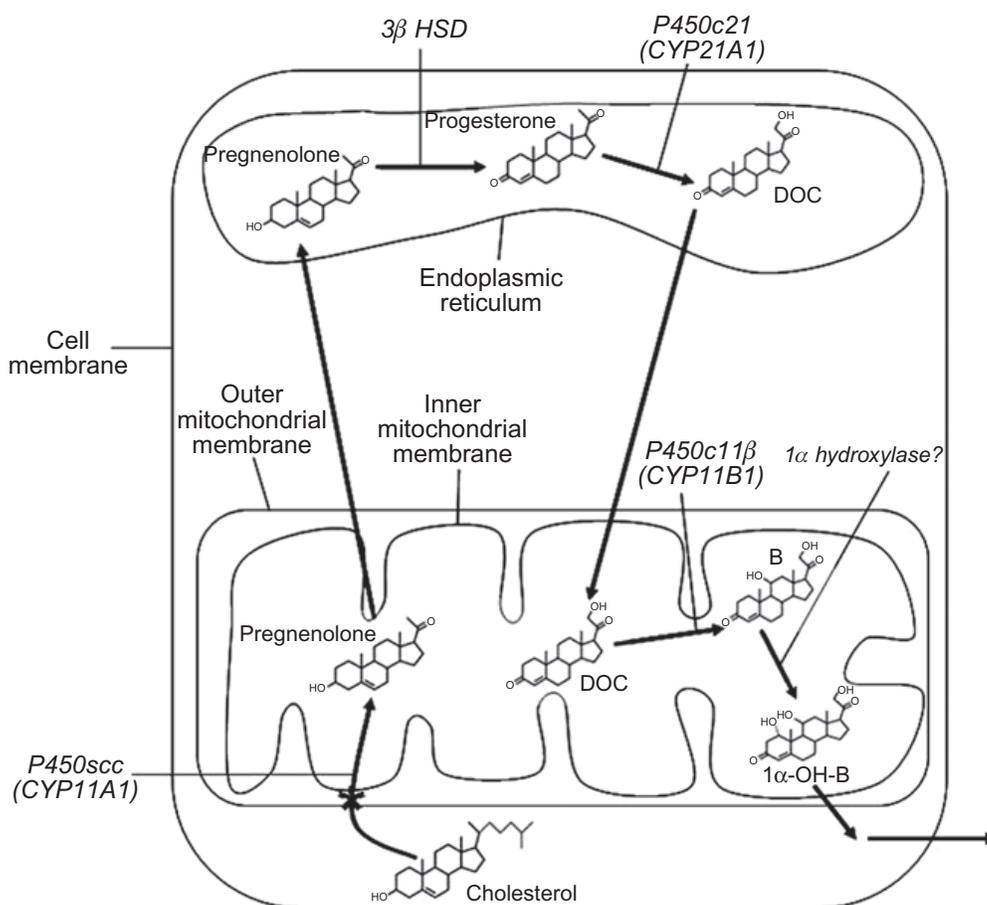


Figure 8.2. Theoretical biosynthetic pathway for 1α -OH-B in interrenal cells of elasmobranchs. P450 and CYP nomenclature is used for the key steroidogenic enzymes. * denotes location of Steroidogenic acute regulatory protein. CYP11B and 1α hydroxylase location and presence is presumed. Figure from Anderson (2012).

mitochondrial membrane by way of the steroidogenic acute regulatory protein (StAR). StAR has been sequenced and cloned from 3 species of ray, the Porcupine River Stingray, *Potomotrygon hystrix*, the South American Freshwater Stingray, *Potomotrygon motoro*, and *D. sabina* (Nunez et al., 2005; Evans and Nunez, 2010). The second step requires the catalysis of cholesterol to pregnenolone, which is achieved by an enzyme in the P450 family, P450 side chain cleavage also known as CYP11A, which has been identified by molecular means in 3 species of ray, the Southern Stingray, *Dasyatis Americana*, *P. motoro*, and *D. sabina* (Nunez and Trant, 1997; Nunez et al., 2006; Evans and Nunez, 2010). CYP17A, 3 β -hydroxysteroid dehydrogenase, and CYP21 are all required for the further conversion of pregnenolone toward the corticosteroids and have all been identified in elasmobranchs (Barry et al., 1993; Nunez and Trant, 1995; Trant, 1995; Nunez and Trant, 1998; Nunez et al., 2006). However, identification of enzymes controlling the final steps in 1 α -OH-B synthesis, such as a putative 1 α -hydroxylase, have yet to be determined in spite of the known existence of this unique steroid for nearly 50 years (Fig. 8.2).

A major benefit of the homologous cell type and discrete anatomy of the interrenal gland in elasmobranchs is that it readily lends itself to *in vitro* perfusion studies (Idler et al., 1967; Idler and Truscott, 1969; Simpson and Wright, 1970; Honn and Chavin, 1978; Hazon and Henderson, 1984, 1985; O'Toole et al., 1990; Armour et al., 1993a; Evans and Nunez, 2010; Evans et al., 2010b). Such preparations are ideal when determining factors involved in regulating corticosteroid release from the interrenal gland. These factors can be broadly categorized dependent on their regulation of energy requirement (frequently glucose) or mineral balance, primarily sodium and potassium. Adrenocorticotrophic hormone (ACTH) (see Section 2.3.1) is well recognized as the major stimulating factor in the vertebrate glucose regulatory response, whereas cortisol (most mammals and fishes), corticosterone (some mammals, amphibians, birds and reptiles), or 1 α -OH-B (elasmobranchs) have all been shown to increase following ACTH stimulus using a variety of *in vivo* and *in vitro* preparations. In early studies using the *in vitro* perfusate preparation mammalian ACTH was shown to increase the concentration of steroid product in the collected perfusate (Macchi and Rizzo, 1962) and this was thought to be a mix of aldosterone, cortisol, and corticosterone depending on the species examined (Bern et al., 1962). We now know that the reported increase in product was most likely 1 α -OH-B and similar studies have indeed demonstrated this. In *S. canicula*, mammalian ACTH stimulated 1 α -OH-B synthesis and secretion in both *in vivo* and *in vitro* preparations (Hazon and Henderson, 1985; O'Toole et al., 1990) likely through an increase in the intracellular second messenger cAMP (Honn and Chavin, 1978; Armour et al., 1993a; Evans and

Nunez, 2010) as shown in mammals (Kojima et al., 1985). Furthermore, molecular expression of both StAR and CYP11A were significantly up-regulated in *D. sabina* interrenal tissue following incubation with 0.5 IU ml⁻¹ of porcine ACTH (Evans and Nunez, 2010).

Aldosterone is the principle corticosteroid in tetrapods responsible for regulating mineral balance with one of the primary functions being renal retention of sodium and excretion of potassium. In mammals, two of the most potent stimuli for aldosterone release are increases in plasma potassium and increases in circulating levels of the antinatriuretic peptide angiotensin II (Ang II) (see Section 6). In nontetrapod vertebrates, the mineral regulating role by cortisol in teleosts, or 1 α -OH-B in elasmobranchs, is in a large part predicated by the stimulus for release and the response is facilitated by the receptor to which the steroid binds (Bury and Strum, 2007). In perfused interrenal glands of *S. canicula* increases of potassium in the perfusion medium were shown to be mildly steroidogenic (O'Toole et al., 1990); however, perfusion with homologous Ang II was shown to be potently steroidogenic in both *S. canicula* and *D. sabina* perfused interrenal gland preparations (Hazon and Henderson, 1985; Armour et al., 1993a; Evans et al., 2010b). Furthermore, perfusion with the natriuretic peptide C-type natriuretic peptide had no effect on 1 α -OH-B production (Evans et al., 2010b). The above perfusion studies suggest potential for stimulus of release of 1 α -OH-B in the whole animal in response to glucose requirement through ACTH or mineral imbalance; however, subsequent physiological studies are less definitive in regard to the ultimate role of 1 α -OH-B.

Evidence supporting a mineral regulating action of 1 α -OH-B has been obtained using heterologous bioassays with 1 α -OH-B stimulating sodium efflux across the toad bladder (Grimm et al., 1969) and sodium retention in the rat (Idler et al., 1967). Interrenalectomy in *D. sabina*, which results in reduced plasma osmolality, provides indirect support for a regulatory role in mineral balance of 1 α -OH-B in elasmobranchs (De Vlaming et al., 1975) and the same procedure in the winter skate, *Leucoraja ocellata*, resulted in reduced NaKATPase activity and sodium output from the rectal gland (Holt and Idler, 1975; Idler and Kane, 1976). More direct support for a mineral regulating role of 1 α -OH-B was provided by Hazon and Henderson (1984) where adaptation of *S. canicula* to salinities below 70% seawater led to preferential retention of sodium and chloride in the plasma over urea and a positive relationship between plasma sodium and 1 α -OH-B concentration. At that time it was unclear if the role of 1 α -OH-B was related to urea or sodium regulation, or both. Subsequent experiments involving dietary protein restriction, thereby limiting urea synthesis, followed by transfer of *S. canicula* to increased salinity (130% seawater), also resulted in a positive relationship between plasma sodium and 1 α -OH-B concentration (Armour

et al., 1993b) strongly supporting a mineral regulating role for 1α -OH-B in elasmobranchs.

There is less empirical support for a glucose regulating role of 1α -OH-B in elasmobranchs and potential reasons are twofold. The first is a technical issue; after supplies from Kime's original synthesis in the 1970s and assay supplies for measurement were exhausted (Kime, 1975; Kime, 1977), manufacturing of the steroid and therefore development of reliable measurement techniques has proven problematic. Indeed, only recently has the steroid become available (Carroll et al., 2008; Evans et al., 2010b). The second is a biological issue, in that elasmobranchs rely heavily on ketone bodies and amino acids as oxidative fuels (see Chapter 7; Speers-Roesch and Treberg, 2010) and reliance on these fuels may confound classical perceptions of glucose regulatory actions of 1α -OH-B. Evidence to support a glucose regulatory role for 1α -OH-B in elasmobranchs is equivocal. Interrenalectomy in 4 species of skate reduced liver glycogen stores but had no effect on circulating glucose levels (Hartman et al., 1944), whereas the same procedure in *L. erinacea* had no effect on liver glycogen stores (Idler et al., 1969) and administration of 1α -OH-B in the rat had no effect on circulating glucose levels. Exposure to capture stress resulted in a mild increase in corticosterone in *S. tiburo* and there was some evidence of seasonal variation of blood corticosterone concentration dependent on reproductive state in *S. tiburo* and *D. sabina* (Manire et al., 2007). These results should be interpreted with caution; however, as the authors indicate interference with 1α -OH-B in their measurement of corticosterone may have confounded the results and therefore interpretation of the data (Manire et al., 2007). Indeed, lack of a homologous assay and the many varied methods used to measure 1α -OH-B make it difficult to draw any cross-species comparisons (Table 8.2).

Upon release lipophilic steroids are typically carried in the plasma to the target site by plasma proteins. These can be specific in nature, such as corticosteroid binding globulin or transcortin in mammals, or general such as plasma albumin proteins. Carrier proteins aid not just as transport proteins but also to reduce enzymatic degradation of the free steroid in the circulation, and further they create an available pool in the circulation where the bound and free fractions exist in a dynamic equilibrium and, as the free fraction is taken up at the target site, additional free steroid is liberated from the carrier protein. In mammals there are two principle carrier proteins, corticosteroid binding globulin (CBG) and serum albumins with the affinity and capacity highest and lowest respectively for CBG. Corticosteroid carrier proteins have been shown to exist in all vertebrates thus far examined and elasmobranchs are no exception (Seal and Doe, 1965; Idler and Freeman, 1968; Freeman and Idler, 1971; Martin, 1975). In teleosts, there appears to

Table 8.2

Measured concentrations of corticosteroids in the body fluid of representative elasmobranchs. Concentrations are in $\mu\text{g}/100\text{ ml}$. Data are presented as a mean \pm sem where possible

| Species | Sex | Source | Cortisol | $1\alpha\text{-OH-B}$ | Corticosterone | Method | Reference |
|---|-------|-------------|-----------------|-----------------------|---------------------|--------|-----------------------------------|
| Sandbar Shark, <i>Carcharhinus plumbeus</i> | ♀ | Plasma | 2.6 | | 5.9 | TLC+F | Phillips (1959) |
| Bull Shark, <i>Carcharhinus leucas</i> | ♀ | Plasma | 2.9 | | 1.7 | | Phillips (1959) |
| | | Plasma | 0.9 | | 1.8 | | |
| Dusky Shark, <i>Carcharhinus obscurus</i> | ♀ | Plasma | 3.3 | | 2.7 | | |
| Spinner Shark, <i>Carcharhinus brevipinna</i> | ♂ | Plasma | 2.9 | | 0.8 | | |
| Tiger Shark, <i>Galeocerdo cuvier</i> | ♀ | Plasma | 1.6 | | 5.2 | | |
| European Dogfish, <i>Scyliorhinus canicula</i> | Mixed | Whole blood | 2.5 | | | TLC+F | Phillips and Chester-Jones (1957) |
| | Mixed | Plasma | | 0.36 ± 0.1 | | RIA | Kime (1977) |
| | ♀ | Plasma | | 3.97 ± 0.28 | | RIA | Hazon and Henderson (1984) |
| | Mixed | Plasma | | ~ 3.6 | | RIA | Hazon and Henderson (1985) |
| | Mixed | Plasma | | ~ 2 | | RIA | Armour et al. (1993a) |
| Spiny Dogfish, <i>Squalus acanthias</i> | Mixed | Plasma | 2.3 ± 0.5 | 1.5 ± 1 | | DIDA | Truscott and Idler (1972) |
| Blue Shark, <i>Prionace glauca</i> | Mixed | Plasma | 0.87 ± 0.05 | 0.3 | | DIDA | |
| Shortfin Mako, <i>Isurus oxyrinchus</i> | Mixed | Plasma | 5.3 | 0.052 | | DIDA | |
| Epaulette Shark, <i>Hemiscyllium ocellatum</i> | Mixed | Feces | | | $*1.2\text{--}20.9$ | HPLC | Karsten and Turner (2003) |
| Whitetip Reef Shark, <i>Triaenodon obesus</i> | ♂ | Plasma | | | 0.03 ± 0.003 | RIA | Rasmussen and Crow (1993) |
| | | plasma | | | 0.01 ± 0.002 | RIA | |
| Lemon Shark, <i>Negaprion brevirostris</i> | ♀ | Plasma | | | ~ 0.04 | RIA | Rasmussen and Gruber (1993) |
| | | Plasma | | | ~ 0.1 | RIA | |
| Bonnethead Shark <i>Sphyrna tiburo</i> | ♂ | | | | ~ 0.245 | RIA | Manire et al. (2007) |
| | | | | | ~ 0.05 | RIA | |
| American Southern Ray <i>Dasyatis americana</i> | ♀ | Plasma | 4.3 | | 4.0 | TLC+F | Phillips (1959) |
| Atlantic Stingray, <i>Dasyatis aabina</i> | ♂ | | | | 0.04 ± 0.008 | RIA | Snelson Jr., et al. (1997) |
| | | | | | 0.01 ± 0.001 | | |

(Continued)

Table 8.2 (Continued)

| Species | Sex | Source | Cortisol | 1 α -OH-B | Corticosterone | Method | Reference |
|--|-------|--------------|----------|------------------|----------------|------------|-----------------------------------|
| Spotted Eagle Ray, <i>Aetobatus narinari</i> | ♂ | Plasma | 6.8 | | 20.4 | TLC+F | Chester-Jones and Phillips (1986) |
| Marbled Electric Ray, <i>Torpedo marmorata</i> | ♀ | Plasma | | 0.082 | | DIDA | Truscott and Idler (1972) |
| Cururu Stingray, <i>Potamotrygon histrix</i> | Mixed | Plasma | | | ~10-~150 | RIA | Brinn et al. (2012) |
| Thorny skate, <i>Amblyraja radiata</i> | Mixed | plasma | | 0.5-2.0 | | TLC +AF | Idler and Truscott (1967) |
| | Mixed | Plasma | | 0.8 \pm 0.4 | | TLC +AF | Idler and Truscott (1968) |
| Thornback Ray, <i>Raja clavata</i> | Mixed | Plasma | | | 8.0 | TLC+F | Phillips and Chester-Jones (1957) |
| | Mixed | plasma | | 4.7 \pm 0.7 | | TLC +AF | Idler and Truscott (1969) |
| | | Perivisceral | | 17 \pm 6.6 | | | |
| | | Pericardial | | 8 \pm 1.2 | | | |
| | | Cranial | | 0.8 \pm 0.4 | | | |
| | Mixed | Plasma | | 3.8 \pm 3.7 | | RIA | Kime (1977) |
| | Mixed | Plasma | | 0.6 \pm 0.08 | 0.021 | DIDA | Truscott and Idler (1972) |
| Winter Skate, <i>Leucoraja ocellata</i> | Mixed | Plasma | | 0.5-2.0 | | TLC +AF | Idler and Truscott (1967) |
| | Mixed | Plasma | | 0.43 \pm 0.1 | 0.18 | DIDA | Truscott and Idler (1972) |
| Barn Door Skate, <i>Raja laevis</i> | Mixed | Plasma | | 0.9 \pm 0.02 | 0.16 | DIDA | |
| Clear Nose Skate, <i>Raja eglanteria</i> | ♀ | Plasma | 5.3 | | | | Phillips (1959) |

~ value estimated from graphical representation of the data; RIA, radioimmunoassay; TLC, thin layer chromatography; F, fluorescence; AF, acid fluorescence; G/LC, gas/liquid chromatography; HPLC, high performance liquid chromatography; DIDA, double isotope derivative assay; 1 α -OH-B, 1 α -hydroxycorticosterone; *, units ng/g of fecal mass.

Table adapted from [Anderson \(2012\)](#).

be two types of corticosteroid carrier protein present; one that is CBG-like with intermediate affinity and abundance for cortisol and one that has relatively low affinity but very high abundance, similar to the binding properties of mammalian serum albumin (Freeman and Idler, 1971; Caldwell et al., 1991). In elasmobranchs, there appears to be only a single carrier protein present that has a low affinity but high capacity for 1α -OH-B similar to that for cortisol and mammalian serum albumin (Freeman and Idler, 1971).

Once at the target site any mineral or glucose regulatory action of 1α -OH-B will be governed by the receptor that the ligand may bind to (Bury and Strum, 2007). A cytosolic receptor for 1α -OH-B was first identified in the gill, kidney, and rectal gland of *L. ocellata* (Idler and Kane, 1980) and more recently both a mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) have been identified by molecular means in *L. erinacea* (Bridgham et al., 2006). Further, Carroll et al. (2008) using a luciferase reporter assay, were able to demonstrate that the putative MR was significantly more sensitive to 1α -OH-B than the putative GR. Therefore, assuming that binding of 1α -OH-B to MR and GR in elasmobranchs induces a 'mineral' and 'glucose' response respectively at the target site, these data suggest that for 1α -OH-B to act effectively to regulate glucose a significantly higher concentration of steroid would be required in the circulation, which is consistent with a stress induced release of corticosteroids.

3.2. Catecholamines

While there are some differences among fishes, the basic components of catecholamine synthesis, degradation, and receptors required for binding and ultimate physiological response at the target site are conserved (for review see Randall and Perry, 1992; Reid et al., 1998). Tyrosine hydroxylase (TH) is responsible for the first and rate-limiting step in dopamine and noradrenaline synthesis; that is, the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA). Aromatic L-amino acid decarboxylase (AADC) then drives the conversion of DOPA to dopamine in the cytosol of the cell with further conversion from dopamine to noradrenaline occurring in storage vesicles catalyzed by the enzyme dopamine- β -hydroxylase (DBH). Finally, the cytosolic enzyme phenylethanolamine-*N*-methyl transferase (PNMT) converts noradrenaline to adrenaline, a *N*-methylated amine (Randall and Perry, 1992; Reid et al., 1998). The conserved nature of this pathway has allowed for the direct transfer of mammalian research tools, such as antibodies raised against TH or biochemical assays developed to measure DBH and PNMT activity, to

better understand the role of noradrenaline and adrenaline in elasmobranch physiology.

Unlike teleosts, the chromaffin tissue in elasmobranchs is anatomically separated from both interrenal and renal tissue. The principle site of synthesis and release by cholinergic stimulation of humorally derived catecholamines in elasmobranchs are the chromaffin cells in the axillary bodies. The axillary bodies are often paired and lie in close association with the cardinal sinus that runs along the dorsal surface of the kidney (Young, 1933; Gannon et al., 1972; Chester-Jones and Phillips, 1986). Both adrenaline and noradrenaline can instantly be released into the circulation that feeds directly into the heart by way of the anterior cardinal sinus and sinus venosus (Gannon et al., 1972; Abrahamson, 1979). In the cardiovascular system, blood vessels supplying the gastrointestinal tract are well innervated by the adrenergic system; however, adrenergic control of the heart is lacking and regulation of systemic vasculature is not significant (Nilsson and Holmgren, 1988). That said, it is important to note that the sympathetic nervous system, although anatomically quite variable remains a source of adrenergic control in the central nervous system of elasmobranchs (Young, 1933; Nilsson, 1983); thus understanding the potential contribution of neurally derived versus humorally derived catecholamines in the control of cardiorespiratory physiology has proven challenging (Farrell and Jones, 1992; Perry and Bernier, 1999).

Unlike teleosts the principle stored catecholamine in elasmobranch chromaffin tissue is noradrenaline (Abrahamson, 1979; Reid et al., 1998), which is reflected in the maximum reported circulating levels of noradrenaline usually being higher than adrenaline and baseline or minimum reported circulating levels varying between noradrenaline and adrenaline (Table 8.3). Once released into the circulation the half-life of catecholamines is short (typically minutes). Degradation is achieved primarily by two enzymes, the mitochondrial enzyme monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO is the principle catabolic agent of neurally derived catecholamines, whereas COMT likely plays a greater role in the breakdown of circulating noradrenaline and adrenaline, although in elasmobranchs the proportional role of COMT or MAO in catecholamine catabolism is not well understood (Mazeaud and Mazeaud, 1973). If the appropriate enzymes are expressed, degradation of catecholamines can occur in any tissue, indeed both are expressed in skeletal muscle, liver, kidney, neural, and interrenal tissue in *S. acanthias* and the Sandbar Shark, *Carcharhinus plumbeus* (Trams and Brown, 1967), and while levels are lowest in skeletal muscle, significantly greater mass of this tissue may result in a proportionally greater contribution to the catabolism of catecholamines. Reduction of circulating catecholamines can also occur through

Table 8.3
Average baseline and peak norepinephrine and epinephrine concentrations (nmol L⁻¹)
in selected species of elasmobranchs

| | Noradrenaline | | Adrenaline | | Stimulus | Source |
|------------------------------|---------------|------|------------|-------|----------------------------|---------------------------|
| | min | max | min | max | | |
| <i>Scyliorhinus canicula</i> | 32 | 447 | 26 | 284 | Acute hypoxia | Butler et al. (1978) |
| | 21 | 63 | 17 | 42 | Chronic hypoxia | Butler et al. (1979) |
| | 14 | 97 | 6 | 96 | Burst swimming | Butler et al. (1986) |
| | 46 | 387 | 62 | 212 | Severe hypoxia | Metcalf and Butler (1989) |
| <i>Squalus acanthias</i> | 30 | 4080 | 55 | 1850 | Head trauma | Abrahamson (1979) |
| | 9 | 53 | 6 | 25 | Capture | Opdyke et al. (1982) |
| | 37 | 863 | 3.65 | 87.43 | Increased K ⁺ | Opdyke et al. (1989) |
| | 3.4 | * | 3.0 | * | Hypoxia/hypercapnia | Perry and Gilmour (1996) |
| | ~6 | ~63 | ~10 | ~165 | Phentolamine and Yohimbine | Bernier et al. (1999) |
| | ~2 | ~48 | ~5 | ~55 | Severe hypoxia | Sandblom et al. (2009) |
| | ~8 | ~120 | ~10 | ~10 | CNP | McKendry et al. (1999) |

*, values not significantly different from baseline; ~, indicates values estimated from graphical representation of the data in the original source. Phentolamine and yohimbine are α -adrenergic receptor antagonists; CNP, C-type natriuretic peptide.

uptake and subsequent storage by cells and this has been demonstrated in the sinus venosus, atrium, and ventricular tissue in the heart of a variety of elasmobranchs (Saetersdal et al., 1975; Jönsson, 1982; Nilsson, 1983). Indeed, measurable levels of DBH activity have been demonstrated in the atrium and ventricle of *E. spinax* (Jönsson, 1982). However, the physiological relevance of those values is uncertain as they were at least two orders of magnitude lower than DBH activities in the axillary bodies for the same species.

Environmental hypoxia, exercise, air exposure, hypercapnia, or acidosis and exposure to ion poor water – in short, stressful environments – are all known to induce an increase in circulating levels of catecholamines in teleosts (for review see Randall and Perry, 1992; Reid et al., 1998; Perry and Bernier, 1999). In elasmobranchs, the most studied has been hypoxia, and the release of catecholamines is thought to target the cardiorespiratory systems to ensure supply and delivery of oxygen to the essential organs (see Chapters 1 and 2). However, the role of humorally versus neurally derived catecholamines in the control of cardiorespiratory responses is not clear. Evidence of a link between circulating catecholamines and ventilatory response in elasmobranchs is equivocal; *S. canicula* exhibiting a high resting

ventilation rate show no ventilatory response to catecholamine administration or hypoxia (Butler and Taylor, 1971; Taylor and Wilson, 1989), whereas those with low resting ventilation rate show a pronounced ventilatory response to catecholamine administration and hypoxia (Metcalf and Butler, 1984; Randall and Taylor, 1991). It was suggested that the fish with high resting ventilation rate may have already been operating at high circulating catecholamine levels and therefore any catecholamine receptors were saturated so additional physiological or endocrine factors would have no further effect (Randall and Taylor, 1991). However, there was no indication of a regulatory role for catecholamines in respiration rate or blood oxygen transfer in hypoxic or hypercapnic *S. acanthias* (Perry and Gilmour, 1996). It is worth noting the distinct species difference between these studies as *S. acanthias* is considerably more active than the more sedentary/benthic dogfish, *S. canicula*.

In many teleost hearts both parasympathetic and sympathetic systems regulate heart rate in the classic “push-pull” arrangement set by the proportion of cholinergic input, inhibiting, and adrenergic input, stimulating, pacemaker activity (Farrell and Jones, 1992). Given the lack of any significant sympathetic regulation, the elasmobranch heart is largely under inhibitory regulation of the vagus nerve, (for review see Nilsson, 1983; Farrell and Jones, 1992; Taylor, 1992; Taylor et al., 2014). Consequently, any significant adrenergic regulation of heart function in elasmobranchs must be humorally derived originating from either the chromaffin cells in axillary bodies lining the cardinal sinus (Gannon et al., 1972; Abrahamson, 1979; Farrell and Jones, 1992) or released from endogenous stores within the heart itself (Saetersdal et al., 1975; Jönsson, 1982). Positive chronotropic (rate of contraction) and inotropic (force of contraction) effects of catecholamines on elasmobranch heart function have been repeatedly demonstrated (MacDonald, 1925; Lyon, 1926; Fänge and Östlund, 1954; Capra and Satchell, 1977a; Van Vliet et al., 1988), and dose-dependent studies using isolated *in vitro* heart preparations from *S. acanthias* show a greater potency of adrenaline over noradrenaline, with physiological concentrations of noradrenaline barely causing a response in the isolated heart preparation (Van Vliet et al., 1988). This leads to the interesting dilemma that if adrenaline is the dominant functional catecholamine why does noradrenaline dominate in storage (Abrahamson, 1979), and in most species, in the circulation (Table 8.3)? To understand this one needs to consider the ligand-receptor interaction.

Originally identified in 1948, α - and β -adrenoreceptors mediate the response of catecholamines at the target site (Ahlquist, 1948). In mammals, numerous isoforms of α_1 (α_{1A} , α_{1B} , α_{1C}), α_2 (α_{2A} , α_{2B} , α_{2C}), β_1 , β_2 , and β_3 (Hieble et al., 1995) have been identified. Adrenaline will preferentially bind

to the α -adrenoreceptor and norepinephrine tends to preferentially bind to the β -adrenoreceptor, and when both are expressed there is often a reversal in the response depending on the dominant catecholamine present. In teleosts, the role and actions of the β -adrenoreceptor have been more fully characterized than the α -adrenoreceptor (Fabbri et al., 1998). Positive chronotropy is thought to be mediated via the β -adrenoreceptor in teleost hearts (Farrell and Jones, 1992), which appears to be most similar to the mammalian β_2 -adrenoreceptor (Ask, 1983). In the elasmobranch heart, positive inotropy and chronotropy has been demonstrated for both noradrenaline and adrenaline in *S. canicula* (Van Vliet et al., 1988). In *S. acanthias* equivalent results were obtained with the exception that noradrenaline-induced negative chronotropy via an α -adrenoreceptor pathway (Capra and Satchell, 1977a). To date, the presence of both α - and β -adrenoreceptors in the cardiorespiratory systems of elasmobranchs is largely based on pharmacological reports using known, mammalian based, α - and β -adrenoreceptor agonists and antagonists. Therefore, while evidence suggests differential release of adrenaline versus noradrenaline from different cell types in the axillary bodies and therefore subsequent differential upstream effects on the cardiorespiratory systems (Capra and Satchell, 1977b), molecular characterization of the adrenoreceptor types and binding kinetics of adrenaline and noradrenaline to those receptors would go a long way in resolving the role of humorally derived catecholamines in elasmobranch cardiorespiratory physiology.

In elasmobranchs there is also a potential regulatory role for catecholamines in salt and water balance. *In vivo* administration of adrenaline in *S. acanthias* resulted in a significant increase in urine flow rate (UFR) alongside an increase in the fractional secretion of urea and other solutes, but no appreciable change in glomerular filtration rate (GFR) (Forster et al., 1972). These results suggest a tubular action of adrenaline in the regulation of renal function independent of haemodynamics. A similar experiment conducted on *S. canicula* showed the adrenaline induced increase in UFR *in vivo* was the result of an increase in GFR, (Brown and Green, 1987), yet when SNGFR was assessed in anaesthetized *S. canicula* water secretion in the distal region of the nephron was evident, which was also reported in *S. acanthias* (Sawyer and Beyenbach, 1985). The mode of action of adrenaline on tubular function is unclear, but it may influence epithelial permeability (Isaia, 1984). Neither adrenaline nor noradrenaline effected secretion rate in the isolated perfused rectal gland of *S. acanthias* (Stoff et al., 1979). However, in the isolated perfused rectal gland in *S. canicula*, addition of noradrenaline restricted perfusion of the gland by up to 95% and the actions were found to be mediated through the α -adrenoreceptors as phentolamine completely reversed the inhibition of flow, furthermore,

similar inhibition of flow was observed in the isolated perfused rectal gland of *S. acanthias* (Shuttleworth, 1983). The observed vascular and epithelial effects of adrenaline and noradrenaline in the kidney and rectal gland of *S. acanthias* and *S. canicula* clearly demonstrate an osmoregulatory role for these catecholamines.

While cholinergic stimulation is likely the major stimulus of catecholamine release from axillary bodies there are additional factors known to stimulate release. Increases in plasma K^+ concentration as a result of intensive exercise has been shown to stimulate catecholamine release in *S. acanthias* (Opdyke et al., 1989); furthermore, there is reported peptidergic regulation of release of catecholamines in fishes by vasoactive peptides such as angiotensin II, natriuretic peptides, vasoactive intestinal peptide (VIP) (Reid et al., 1998) and possibly neuropeptide Y (Chiba, 2001).

Both corticosteroids and catecholamines are commonly linked when fish are responding to a stressful event as both will work to mobilize energy reserves and fuel the fight or flight response and recovery (Wendelaar Bonga, 1997). Both noradrenaline and adrenaline have been shown to stimulate plasma glucose concentration in two species of elasmobranch *in vivo* (De Roos and De Roos, 1978), but empirical evidence of an increase in glucose in the circulation of elasmobranchs following a stressful event is again equivocal with some showing increases (Manire et al., 2001; Brooks et al., 2012) and some not (Cicia et al., 2012; Frick et al., 2012) possibly due to the potential for alternative energy use (Speers-Roesch and Treberg, 2010) or variation in nutritional states. In elasmobranchs there is evidence to suggest regulatory control of interrenal output by catecholamines (Trams and Brown, 1967); however, the possible glucose regulatory role of 1α -OH-B is uncertain and the interrenal response in elasmobranchs has been described as sluggish (Chester-Jones and Phillips, 1986). These factors, combined with the observations that in order to elicit a catecholamine response to hypoxia the exposure must be severe (Butler et al., 1978, 1979) suggests that what we understand as the classic endocrine stress response from research on teleosts may not fit elasmobranchs, thus elasmobranchs are clearly a group of fishes ripe for research understanding the evolutionary basis of the endocrine stress response in vertebrates.

4. GASTRO-ENTERO-PANCREATIC HORMONES

4.1. Intestinal Hormones

The gut is both well innervated (Holmgren and Nilsson, 1983) and vascularized with the mesenteric, lineogastric, and coeliac arteries being

the main vessels branching from the dorsal aorta supplying the gastrointestinal tract. The enteric nervous system in fishes was recently reviewed (Olsson, 2011) and many of the classic vertebrate gut hormones have been identified in elasmobranchs either indirectly by immunostaining techniques using heterologous antibodies, or directly by biochemical extraction or molecular means. Somatostatin (SST) (Conlon et al., 1985); neuropeptide Y and peptide YY (NPY, PYY) (Conlon et al., 1991; Pan et al., 1992); VIP (Dimaline and Thorndyke, 1986); serotonin (El-Salhy, 1984); cholecystikinin (CCK) (Bjenning and Holmgren, 1988); the tachykinins, scyliorhinin I and II, neurokinin A and substance P (Conlon et al., 1986); bombesin; and catecholamines (Holmgren and Nilsson, 1983) are some of the major neuropeptides identified. Many of these neuropeptides have also been identified in discrete endocrine cells lining the gut (Table 8.4) either independently or co-localized (Cimini et al., 1989; Chiba, 1998), although the physiological relevance of co-localization is unknown (Holmgren and Nilsson, 1999).

The traditional pancreatic hormones, insulin, glucagon, pancreatic polypeptide family, and SST are also found in endocrine cells lining the intestine (El-Salhy, 1984; Cimini et al., 1992; Reinecke et al., 1992; Falkmer, 1995; Holmgren and Nilsson, 1999) alongside immunoreactive cells for gastrin and CCK (Jönsson, 1995) and are summarized in Table 8.4. The vast majority of studies to date have focused on identification and location of the gastro-entero-pancreatic hormones in elasmobranchs. Therefore, our understanding of the role these hormones play in elasmobranch physiology is growing with most studies examining key functional aspects of the intestine such as gastric acid secretion by gastrin (Hansen, 1975; Vigna, 1983) and gallbladder contractility by gastrin and CCK (Andrews and Young, 1988b).

Stimulatory actions of neurotransmitters and hormones on gut motility such as stomach contraction by adrenaline (Holmgren and Nilsson, 1983) and intestinal smooth muscle activity by CCK, gastrin, and bombesin (Nilsson and Holmgren, 1988; Aldman et al., 1989) have been demonstrated alongside potential inhibitory neuropeptides such as VIP (Nilsson and Holmgren, 1988). Regulation of blood flow to the gut is key for normal function and elasmobranchs are no exception in this regard with catecholamines and VIP demonstrating inhibitory actions on blood flow to the intestine (Holmgren et al., 1992b) and NPY, bombesin, and substance P increasing blood flow to the intestine (Bjenning et al., 1990; Holmgren et al., 1992a). It is important to note that the observed increase in gut blood flow following NPY administration was due to a decrease in vascular resistance in the intestinal blood vessels, whereas the increase following bombesin administration was due to an increase in vascular resistance of the somatic vasculature (Holmgren et al., 1992a,b), which demonstrates a key

Table 8.4
Gastro-entero-pancreatic hormones identified in representative elasmobranchs and demonstrated physiological function.

| | Immunoreactivity | | Sequence known ^c | Reported physiological action |
|-------------------------------|------------------|----------|-----------------------------|--|
| | gut | pancreas | | |
| Insulin | Yes | Yes | Yes | Hypoglycemic |
| Glucagon | Yes | Yes | Yes | Hyperglycemic, stimulates RG secretion ^b |
| Somatostatin | Yes | Yes | Yes | Inhibits RG secretion |
| Pancreatic polypeptide | | Yes | No | |
| Neuropeptide Y | Yes | Yes | Yes | Vasoactive, inhibits RG secretion, orexigenic ^c |
| Vasoactive Intestinal Peptide | Yes | Yes | Yes | Vasoactive, stimulates RG secretion ^c |
| Cholecystokinin | Yes | Yes | Yes | Myoactive |
| gastrin | Yes | Yes | Yes | Myoactive |
| Tachykinins | | | | |
| Neurokinin A | Yes | | Yes | |
| Substance P | Yes | | Yes | Vasoactive |
| Scyliorhinin I | Yes | | Yes | Vasoactive |
| Scyliorhinin II | Yes | | Yes | Stimulates RG secretion |
| Gastrin releasing peptide | Yes | | | |
| Bombesin | Yes | | | Vasoactive |
| Galanin | Yes | | | Vasoactive |
| Ghrelin | Yes | | Yes | Stimulated GH receptor assay |
| C-type natriuretic peptide | Yes | | Yes | Stimulates RG secretion, vasoactive, inhibits drinking |
| Adrenomedullin | | Yes | No | |

RG, rectal gland; Vasoactive, peptide influences contractility of vascular smooth muscle; Myoactive, peptide influences contractility of smooth muscle (eg: intestine or gallbladder). Immunoreactivity in the intestine includes immunoreactivity associated with the mucosa and enteric nerves supplying the gut. Pancreatic immunoreactivity is associated with islet cells.

^aSequence includes either the gene or amino acid sequence.

^bResults are variable between species (see text for details).

^cAction based on changes in gene expression.

difference in the location and expression of receptors for these two neuropeptides.

4.1.1. GASTRIN/CHOLECYSTOKININ

Gastrin and CCK were originally identified based on their respective abilities to stimulate gastric acid and gallbladder ejection of bile in mammals (Edkins, 1905; Ivy and Oldberg, 1928). It is now recognized that gastrin and CCK are related hormones with an ancient origin that share a common

amino acid sequence at the C-terminal end of the peptide hormone (Dupré and Tostivint, 2014). Both gastrin and CCK-like immunoreactivity have been identified in the intestinal epithelia and pancreas of elasmobranchs (Tagliaferro et al., 1985; Jönsson, 1995) and separate genes for gastrin and CCK have been sequenced in *S. acanthias* and the porbeagle, *Lamna cornubica* (Johnsen et al., 1997). There are numerous variants of these hormones typically with N-terminus extensions; and posttranslational sulfation of a tyrosine residue close to the C-terminus of CCK significantly influences binding affinity of the hormone to its receptor (Johnsen et al., 1997; Rehfeld et al., 2007). At least four forms of CCK and five forms of gastrin have been identified in mammalian plasma (Rehfeld et al., 2007) and three forms of gastrin and two forms of CCK were identified in the brain and intestine of *S. acanthias* and *L. cornubica* (Johnsen et al., 1997). Despite this variation, administration of mammalian variants of gastrin and CCK contracted isolated gallbladder strips from three species of ray (Andrews and Young, 1988b). However, even though high concentrations of gastrin and CCK-like substances were obtained from the rectum, results were equivocal regarding a potential vasoactive role in isolated rectum muscle strips from *S. acanthias* (Aldman et al., 1989) and, while gastrin increased motor activity of muscle strips from the cardiac stomach, it was without effect on isolated muscle strips from the rectum in three species of ray (Andrews and Young, 1988a). Given the variability of the gastrin/CCK peptides there is clearly a substantial *in vitro* research effort required to understand the bioactivity of this family of hormones in elasmobranchs.

4.1.2. TACHYKININS

Tachykinins are a group of neuropeptides that include neurokinins, substance P, neuropeptide γ , and neuropeptide K that share a structural similarity at the C-terminus of the peptide and typically induce a contractile response in intestinal smooth muscle (Conlon, 1999). Two additional tachykinins, scyliorhinin I and II, have been isolated from the gut of *S. canicula* based on their contractile properties on isolated longitudinal smooth muscle from the guinea pig ileum (Conlon et al., 1986). A substance-P-like peptide has been isolated from the brain of *S. canicula* (Vaugh et al., 1993) and a neurokinin-A-like peptide has been isolated from the brain of *R. rhina* (Vaugh et al., 1994). Tachykinin-like receptors have been found throughout the gastrointestinal tract of elasmobranchs in addition to nerve fibers synapsing with cardiac and vascular tissue (Conlon, 1999). Both scyliorhinin I and substance P have been shown to induce a vasodepressor response in *S. canicula* (Vaugh et al., 1993) and scyliorhinin I increased blood flow to the intestine of *S. acanthias* via a reduction in vascular

resistance of the intestinal blood vessels (Kågström et al., 1996). Vasoactive Intestinal Peptide (VIP) is a potent stimulatory agent for rectal gland secretion in *S. acanthias* (Stoff et al., 1979), but does not stimulate secretion from the isolated perfused rectal gland of *S. canicula* (Shuttleworth, 1983). Subsequent investigations partially purified an alternative hormone named rectin from the gut of *S. canicula* that stimulated chloride secretion from the rectal gland (Shuttleworth and Thorndyke, 1984), this was later identified as the tachykinin scyliorhinin II (Anderson et al., 1995). The current hypothesis for the role of gut hormones in regulating rectal gland secretion is that the gorge feeding frequently observed in a number of elasmobranchs results in the potential for significant salt loading. This may stimulate the release of scyliorhinin II and/or VIP that enhances chloride secretion from the rectal gland increasing salt excretion and a return to salt balance (see Chapter 5; Stoff et al., 1979).

4.1.3. VASOACTIVE INTESTINAL PEPTIDE

VIP was first identified from the small intestine in mammals because of its potent vasodilator ability (Said and Mutt, 1970) and was subsequently identified throughout the central nervous system (Said and Rosenberg, 1976). Since this discovery it is now recognized that VIP belongs to the secretin-family of peptide hormones, acts primarily as a neurotransmitter/neuromodulator in mammals, and is involved in regulation of a number of physiological systems including the cardiovascular, respiratory, renal, and reproductive systems (Henning and Sawmiller, 2001). In elasmobranchs, VIP-like immunoreactivity has been identified in non-neural tissue throughout the gastrointestinal tract as well as perivascular nerve fibers supplying the coeliac and mesenteric arteries (Holmgren and Nilsson, 1983).

In vivo administration of porcine VIP in *S. acanthias* resulted in an increase in dorsal aortic pressure, heart rate, and resistance to blood flow in the coeliac arterial bed of the gut in *S. acanthias*, but had no effect on somatic vasculature (Holmgren et al., 1992b). The increase in resistance was unusual as VIP is vasodilatory in mammals (Henning and Sawmiller, 2001). The most intensive line of research for VIP in elasmobranchs has been its regulatory role of rectal gland function. In 1979, Stoff and colleagues had established an isolated perfused rectal gland preparation from *S. acanthias* and examined the potential action of a host of hormones to determine the regulatory factors involved in rectal gland function (Stoff et al., 1979). From that study an inhibitory role for SST and a stimulatory role for VIP were established. Later, volume loading was found to significantly stimulate rectal gland secretion and the humoral factor implicated was VIP (Solomon et al., 1984). Subsequent investigations determined that the humoral substance was not VIP but the natriuretic peptide C-type natriuretic peptide

(CNP) released from the heart in response to volume loading (Solomon et al., 1992). CNP then stimulates the release of VIP (Silva et al., 1987) from localized neurons innervating the gland (Holmgren and Nilsson, 1983; Stoff et al., 1988). The increase in local VIP then stimulates secretion of salt through activation of the sVIP receptor on the epithelia cells (Bewley et al., 2006). The most curious aspect of the role of VIP in rectal gland secretion is that thus far, VIP only stimulates secretion from *S. acanthias* and fails to induce a response in *S. canicula*, *L. erinacea*, and *L. clavata* (Shuttleworth, 1983; Shuttleworth and Thorndyke, 1984; Kelley et al., 2014), this despite the VIP-like substance identified in both *S. acanthias* and *S. canicula* (Dimaline and Thorndyke, 1986). For more in depth description of salt and water balance and the role of the rectal gland and kidney the reader is referred to Chapter 5.

4.1.4. GHRELIN

Ghrelin, first isolated from the stomach of rats for its stimulatory action on growth hormone release (Kojima et al., 1999) has since been identified in a variety of vertebrates with physiological function including regulation of growth hormone release, orexigenic activity, and inhibition of drinking (Kaiya et al., 2013). A ghrelin-like peptide has been identified from the stomach of the hammerhead shark, *Sphyrna lewini*, and the Black tip Reef Shark, *Carcharhinus melanopterus*, (Kawakoshi et al., 2007) and from *D. akajei* (Kaiya et al., 2009). mRNA expression was highest in the stomach of *S. lewini* and *D. akajei* and interestingly high mRNA expression was also observed in the rectal gland of *D. akajei*. In all three species the peptide was able to activate mammalian growth hormone secretagogue receptor (Kawakoshi et al., 2007; Kaiya et al., 2009) which suggests a similar function as identified in mammals; however, further studies are required to elucidate the true physiological function of ghrelin in elasmobranchs.

4.1.5. OTHER GASTRO-ENTERO-PANCREATIC HORMONES

Additional peptides such as gastrin releasing peptide (GRP) (Conlon et al., 1987) and bombesin (Bjening et al., 1990) have been identified in the elasmobranch gut and bombesin was shown to increase blood flow to the intestine (Bjening et al., 1990; Holmgren et al., 1992a). However, it is worth noting that despite the observed vascular effects of bombesin in elasmobranchs, the presence of this peptide is based on immunoreactivity using heterologous antibodies and the hormone remains to be characterized. Indeed, it was suggested that bombesin may not even exist in elasmobranchs (Conlon et al., 1987). Galanin, a neuropeptide/neuromodulator in mammals, has been characterized from the gut of *S. canicula* (Wang and Conlon, 1994). Galanin-like immunoreactivity was found throughout the brain of

S. canciula, which suggests a similar neuromodulatory role (Vallarino et al., 1991) and also found surrounding gut blood vessels in two species of shark and one species of ray. Porcine galanin caused an increase in blood pressure in the sharks, but not the ray, and caused vasoconstriction in the pancreatico-mesenteric artery from all three species (Preston et al., 1995). At present these are the only studies demonstrating a physiological role for galanin, bombesin, and GRP in elasmobranchs. However, given the immunoreactivity of all three peptides in higher neural centers and additional functional roles such as regulation of food intake for galanin and bombesin in fish (Volkoff et al., 2009), it is likely that further physiological roles for these hormones will be revealed. Significant amounts of C-type natriuretic peptide have also been identified throughout the digestive tract in *T. scyllia* (Suzuki et al., 1994). The physiological relevance of this in elasmobranchs is as yet unexplored, but may be involved in regulating NaCl and water transport across the intestine as described in teleosts (Ando et al., 1992). Adrenomedullin-like immunoreactivity was demonstrated in the pancreas of *C. plumbeus* where it was co-localized in pancreatic islet cells with insulin-like, glucagon-like, and pancreatic polypeptide-like immunostaining but never by itself (López et al., 1999). In teleosts, adrenomedullin has been shown to be involved in regulation of food intake and drinking (Takei and Loretz, 2011); however, a physiological role for this peptide hormone in elasmobranchs has yet to be reported. Finally, guanylin belongs to a family of peptides, including guanylin, renoguanylin, and uroguanylin (Cramb et al., 2005) and an osmoregulatory role has been established for guanylin in teleosts in the regulation of water and chloride movement in gut epithelia (Takei and Loretz, 2011). The peptide has yet to be identified in elasmobranchs, but human guanylin stimulated chloride secretion in the perfused rectal gland of *S. acanthias* (Silva et al., 1997), although it had no effect on short circuit current in isolated rectal gland tubules from the same species (Greger et al., 1999). Why this discrepancy exists between preparations is unexplained but may be the result of a guanylin having a vascular effect on the rectal gland as opposed to a direct action on secretory epithelia.

4.2. Pancreas

Elasmobranchs have a bi-lobed pancreas with a single pancreatic duct that empties into the anterior portion of the intestine just posterior to the pyloric sphincter. Despite the ease of cannulation of the pancreatic duct and the vascular supply to the pancreas, few studies have examined the regulatory factors controlling pancreatic exocrine activity with a suggestion that acidic chyme in the duodenum may influence secretion of pancreatic

juice into the intestine (Babkin, 1929). One hormone that may regulate exocrine activity is secretin as originally described by Bayliss and Starling using a heterologous assay (Bayliss and Starling, 1903); however, while Bayliss and Starling identified a secretin-like response in the dog over 110 years ago, the equivalent experiments on an elasmobranch remain to be reported. VIP purified from the elasmobranch intestine has been shown to stimulate pancreatic juice secretion from the turkey (Dimaline and Thorndyke, 1986), but effects on the elasmobranch pancreas have not been reported despite our understanding that VIP and secretin belong to the same family of peptide hormones (Mommsen and Busby, 2006; Roch et al., 2011).

Pancreatic islets in elasmobranchs have been termed as ‘mammalian-type’ (Falkmer, 1995) and contain A (glucagon secreting), B (insulin secreting), D (somatostatin secreting), and F cells (Pancreatic polypeptide/neuropeptide Y secreting) (Youson and Al-Mahrouki, 1999; Takei and Loretz, 2011). Although recent transcriptome data from the pancreas of *S. canicula* suggest the identification of pancreatic polypeptide may have been the result of antibodies used in immunostaining cross reacting with either NPY or PYY (Mulley et al., 2014). Immunoreactivity for all of the classic pancreatic hormones in addition to a number of other well-recognized gastro-entero-pancreatic hormones has been reported in a number of elasmobranch species (Kobayashi and Ali, 1981; Jönsson, 1991). Islet morphology and association with pancreatic ducts shows considerable species variation (Youson and Al-Mahrouki, 1999) with as many as eight different endocrine cell types being identified (Kobayashi and Ali, 1981). For detailed diagrammatic representation of pancreatic morphology and islet distribution see two previous volumes of this series, Youson, 2007 and Takei and Loretz, 2011. A significant amount of research has been conducted on the endocrine output from the elasmobranch pancreas and digestive tract (El-Salhy, 1984; Cimini et al., 1989; Chiba et al., 1995; Youson and Al-Mahrouki, 1999), so here we will focus on homologous hormones that have been identified and sequenced in elasmobranchs and what is known regarding their output and physiological actions.

4.2.1. INSULIN

The hypoglycemic action of insulin is likely one of the most widely recognized hormonal functions in vertebrates and since its discovery in 1922 (Banting et al., 1922), insulin has been perhaps one of the most intensely studied hormones. The mature peptide consists of an A and B chain joined by invariant cysteine residues that form di-sulphide bridges between positions A7–B7 and A20–B19 with a third di-sulphide bond between

A6–A11 (for review see Conlon, 2001). Insulin has been identified in 4 species of elasmobranch, *T. marmorata* (Conlon and Thim, 1986), *S. acanthias* (Bajaj et al., 1983), Hammerhead Shark (*Sphyrna lewini*), and *S. canicula* (Anderson et al., 2002a) and there is a high degree of homology between both the A chain (~90%) and B chain (~87%) across all four species. Unusually elasmobranchs have a single glutamine C-terminus extension on the A chain compared to other vertebrates (Conlon, 2001). Using heterologous binding assays, binding affinity of *S. acanthias* insulin was lower than bovine insulin to the bovine insulin receptor (Bajaj et al., 1983), but insulin purified from *S. canicula* bound to human recombinant insulin receptors with higher affinity than human insulin (Anderson et al., 2002a). It was suggested that the substitution of arginine for threonine at position 8 in the A chain stabilized the ligand-receptor interaction (Kaarsholm et al., 1993) and thus resulted in a greater binding affinity.

From a functional perspective potential hypoglycemic actions of insulin are naturally the most intuitive line of investigation; however, in comparison to mammals, teleosts are relatively intolerant to glucose (Moon, 2001), and with a predominantly carnivorous diet, blood amino acid concentration maybe a more potent stimulus for insulin release (Navarro et al., 2006). At present there are no reports describing the mechanism for insulin release in elasmobranchs; however, based on a mammalian assay for insulin, circulating levels of the hormone were found to vary seasonally in *S. acanthias* with peak levels occurring during pre-spawn periods and the active feeding period when the hepato-somatic index was rapidly increasing later in the year (Gutiérrez et al., 1988). Interestingly the highest peak in circulating levels of insulin coincided with the lowest circulating levels of glucose; however, it must be noted that the assays used to measure circulating levels of insulin were heterologous mammalian based assays, so while relative values may hold true, absolute values may not be reflective of those reported (Gutiérrez et al., 1986; Gutiérrez et al., 1988). Nonetheless, a number of studies have shown a prolonged hypoglycemic response of up to 7 days, which followed administration of both heterologous and homologous insulin (Grant, 1964; Patent, 1970; De Roos and De Roos, 1979; De Roos et al., 1985; Anderson et al., 2002a) alongside an increase in muscle glycogen deposition (Patent, 1970) in elasmobranchs. The reliance on ketone bodies as an alternative aerobic fuel source in elasmobranchs (see Chapter 7 Zammit and Newsholme, 1979; Speers-Roesch and Treberg, 2010; Polakof et al., 2011) has also prompted measurement of β -hydroxybutyrate and acetoacetate following insulin administration, though the hormone was without effect on circulating levels of either of these energy sources (De Roos et al., 1985; Anderson et al., 2002a).

The sluggish nature of the hypoglycemic response requires further examination. In fed and fasted elasmobranchs, glucose levels are reasonably stable (De Roos et al., 1985; Walsh et al., 2006) suggesting regulation of glucose, with the hypoglycemic insulin being an obvious candidate. In mammals expression of GLUT2 on pancreatic islet B cell membranes mediates insulin release in response to an increase in circulating glucose (for review see Polakof et al., 2011). Insulin sensitive GLUT4 facilitates rapid glucose uptake by peripheral tissues and GLUT4 has been identified in the muscle tissue of teleosts (Capilla et al., 2002). In elasmobranchs three studies thus far have identified glucose transporters. GLUT1 has been identified in the blood brain barrier and choroid plexus (brain cerebral spinal fluid barrier) of two species of elasmobranch the Pacific Sea Shark, *Schroederichthys chilensis*, and *S. canicula* (Balmaceda-Aguilera et al., 2012). Interestingly, this location is consistent with what is seen in mammals for this transporter. Further, a Na⁺-glucose transporter has been identified in the kidney of *S. acanthias* (Althoff et al., 2006) and the kinetics of this transporter support the original hypothesis of no measurable transport maxima for glucose in the shark kidney (Boylan and Antkowiak, 1966). GLUT1 and the insulin sensitive GLUT2 have been detected in the pancreatic transcriptome of *S. canicula* (Mulley et al., 2014). Alternative tissue expression of these glucose transport mechanisms is not known and identification of, in particular, insulin sensitive GLUT4 remains to be reported in elasmobranchs. In regard to a role in amino acid regulation, insulin was without effect on alanine uptake in isolated hepatocytes from *L. erinacea* (Ballatori and Boyer, 1988), despite a significant decrease in plasma alanine concentration in *S. acanthias* following administration of insulin *in vivo* (De Roos et al., 1985). Further, specific glucose uptake mechanisms and or anabolic effects of insulin on protein or lipids in elasmobranchs are as yet undescribed. The latter would be of particular interest given the high lipid content of the elasmobranch liver (Olivereau and Leloup, 1950).

4.2.2. GLUCAGON

Released from A cells in the mammalian pancreas, glucagon belongs to a family of peptides known as the secretin super family and includes, but is not limited to, secretin, glucagon, glucagon-like peptide-1 and 2 (GLP 1 & 2), growth hormone releasing hormone (GHRH), VIP, parathyroid hormone (PTH), parathyroid hormone related protein (PTHrP), and pituitary adenylate cyclase activating peptide (PACAP) (Mommensen and Busby, 2006). The classic mammalian role for glucagon is antagonistic to the effects of insulin, that is it acts in a hyperglycemic fashion, and a similar mode of action has been demonstrated in teleosts (Moon, 2004). Glucagon was first identified from the pancreas of *T. marmorata*, (Conlon and Thim, 1985)

then subsequently two molecular forms of glucagon and one GLP-1 were identified from the pancreas in *S. canicula* (Conlon et al., 1994), which showed a high degree of conservancy in the amino acid structure of glucagon across vertebrates. Reports examining the physiological effects of glucagon on elasmobranchs are few. A mild but transient hyperglycemia was observed following *in vivo* administration of bovine glucagon in *S. acanthias* and *L. erinacea* (Grant, 1964; Patent, 1970) with no effect on liver glycogen stores (Patent, 1970) or alanine transport in isolated hepatocytes (Ballatori and Boyer, 1988). Most recently an intriguing role for glucagon was demonstrated as a secretagogue for the rectal gland of *L. erinacea* but not *S. acanthias* (Kelley et al., 2014). In *S. canicula*, glucagon was also found to stimulate rectal gland secretion *in vitro* (Fig. 8.3) and interestingly, as with *L. erinacea*, VIP was without effect in the rectal gland of *S. canicula* (Shuttleworth and Thorndyke, 1984; Anderson et al., 1995). VIP is one of the most potent chloride secretagogues in the rectal gland of *S. acanthias* (Stoff et al., 1979) so this species difference remains to be resolved. Circulating levels for glucagon were reported for *S. canicula*,

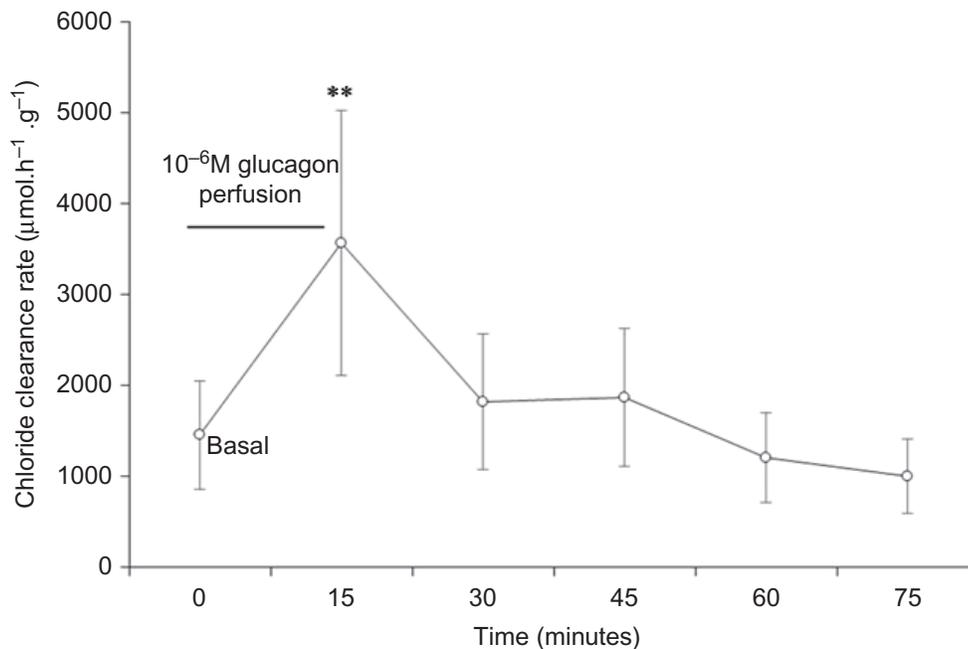


Figure 8.3. Effect of perfusion of 10^{-6} M homologous glucagon on chloride secretion rate in the isolated perfused rectal gland of *S. canicula*. Baseline secretion rate was collected for 15 min prior to perfusion of homologous glucagon. Perfusion medium was elasmobranch Ringers (see Anderson et al., 1995 for recipe). Values are expressed as clearance rate of chloride in $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1} \pm \text{SEM}$, ($N = 6$), ** represents significant difference from baseline value (repeated measures ANOVA). Data from Anderson, Hazon, and Conlon, unpublished.

and found to be extremely low; however, as for the insulin assay described above the measurements were made using a mammalian based heterologous assay so interpretation of values must be taken with caution (Gutiérrez et al., 1986).

4.2.3. SOMATOSTATIN

As the name suggests somatostatin (SST) is involved in the regulation of growth and was originally identified based on its ability to inhibit the release of growth hormone from the pituitary in mammals (Brazeau et al., 1973). Somatostatin-like immunoreactivity was first reported in the intestine of elasmobranchs (Vale et al., 1976) and subsequently purified and sequenced from the pancreas and gut of *T. marmorata* (Conlon et al., 1985). Six genes encoding for pro-somatostatin (PSS) have been identified in teleosts (*SS 1–6*) (Liu et al., 2010), and three have been identified in elasmobranchs (*SSa, b, and c*), with the *SSa* and *c* genes being most similar to the *SS1* and 2 genes in teleosts (Quan et al., 2013). Depending on the extent of post-translational modification from the pro-somatostatin peptide (of which there are two variants PSS-1 and PSS-2) somatostatin will exist as the short form SST-14 or N-terminus extended forms that vary considerably in length depending on species, tissue, and PSS (Nelson and Sheridan, 2006). The short form SST, SST-14, was isolated and sequenced from *T. marmorata* and was found to be the only variant of the peptide hormone in the brain, stomach, pancreas, and intestine, which suggests a distinct lack of post-translational modification of SST in elasmobranchs (Conlon et al., 1985). A putative SST-14 was also predicted from an EST database of *L. erinacea* (Liu et al., 2010) and found to be 72% identical to *T. marmorata* but only 1 amino acid different (93% identity) from SST-14 in the holocephalan elephantfish, *Calorhynchus milii* (Liu et al., 2010). The reported difference between SST-14 in *L. erinacea* and *T. marmorata* is unusual in that SST-14 at the time of identification in *T. marmorata* was identical in all vertebrates (Conlon et al., 1985). Subsequent molecular identification of three somatostatin variants from *S. canicula*, revealed two have the same molecular sequence as *T. marmorata* and one may be more subject to post-translational N-terminus extension as observed in a number of teleosts (Quan et al., 2013). Differential expression in a variety of tissues of all three variants suggests distinct physiological roles for these peptide hormones. However, there are few physiological studies examining a role for somatostatin in elasmobranchs, although it has been shown to inhibit chloride secretion from the rectal gland in *S. canicula* (Stoff et al., 1979; Silva et al., 1985) and its release from nerve terminals may be mediated by the neurotransmitter bombesin (Silva et al., 1990).

4.2.4. NEUROPEPTIDE Y

Neuropeptide Y, first identified from the porcine brain (Tatemoto et al., 1982), belongs to a family of neuropeptides that includes pancreatic polypeptide (PP) and polypeptide YY (PYY). Within elasmobranchs it was first identified in the brain of *S. canicula* (Vallarino et al., 1988) and has subsequently been shown to be present in the brain, gut, pancreas, and vasculature of a variety of different elasmobranch species (Holmgren and Nilsson, 1983; Conlon et al., 1991, 1992a; Cimini et al., 1992; Pan et al., 1992; Bjenning et al., 1993a; McVey et al., 1996; Chiba, 1998). There are two subfamilies of G-protein coupled NPY receptors in mammals, Y1 and Y2. The Y1 subfamily has Y1, 4, and 6 subtypes and the Y2 subfamily has the Y2 and 5 subtypes (Larhammar et al., 2001). The Y1 subfamily is thought to regulate post-junctional effects when NPY is released from the nerve terminal and the Y2 receptor is thought to modulate release from the post-synaptic terminal (Grundemar and Hakanson, 1993). A single receptor most analogous to the Y1 receptor has been identified in the brain of the smooth dogfish, *Mustelus canis* (McVey et al., 1996) and subsequently 3 genes – each encoding for a mammalian Y1 subfamily NPY receptor, Y1, Y4, and Y6 – were shown to be differentially expressed in various tissues of *S. acanthias*, which suggests early duplication of this family of receptors and subsequent divergence of function (Salaneck et al., 2003). NPY is one of the more widely studied of all the gastro-entero-pancreatic hormones in regard to elasmobranch physiology. It is known to be involved in a number of physiological processes such as regulation of blood pressure through its action on vascular contractility (Bjenning et al., 1993a,b; Preston et al., 1998); osmoregulation through its action on the rectal gland (Silva et al., 1993); and food intake through its action on potential hypothalamic feeding areas (Demski, 2012; Hoskins and Volkoff, 2012) and it may also influence catecholamine secretion from the axillary bodies (Chiba, 2001).

NPY-like immunoreactivity has been identified in the rectal gland of *S. acanthias* (Holmgren and Nilsson, 1983) and it was shown to inhibit VIP stimulus of chloride secretion from the isolated perfused rectal gland of *S. acanthias* by as much as 63% (Stoff et al., 1979). The inhibitory action of NPY is thought to be downstream of cAMP production as NPY was ineffective at inhibiting adenylate cyclase, but was effective at inhibiting the stimulatory actions of exogenous forskolin and cAMP (Silva et al., 1993). Dogfish NPY and PYY have both been shown to act as vasopressors on the isolated afferent branchial artery in a dose-dependent manner in *S. canicula* (Bjenning et al., 1993a). A similar result was observed using an isolated pancreatico-mesenteric artery in the Port Jackson Shark, *Heterodontus portusjacksoni*, the Epaulette Shark, *Hemiscyllium ocellatum*, and the Giant Shovelnose Ray, *Rhinobatos*

typus (Preston et al., 1998). Furthermore, dogfish NPY and PYY produced a pronounced vasopressor response *in vivo* in these three species (Preston et al., 1998). The *in vitro* response reported in elasmobranchs is vessel-specific as no response was seen in the efferent branchial artery in the study conducted by Preston et al. (1998), further it appears that the action of NPY is likely mediated through a Y1-like receptor as specific mammalian Y1 antagonists blocked the effect in *S. canicula* (Bjenning et al., 1993a). Lastly the action of NPY may not require an intact endothelium as vasoconstriction was still observed in denuded isolated afferent branchial arteries of *S. canicula* and coronary arteries of *R. rhina* (Bjenning et al., 1993a,b). The vasoconstrictive actions of NPY both *in vivo* and *in vitro* are counter to the observed reduction in coeliac vascular resistance in *S. acanthias* following NPY administration *in vivo* (Holmgren et al., 1992a).

In regard to the regulation of feeding, while there is a host of studies on the neuro-endocrinology of feeding in teleosts and NPY has been shown to be potently orexigenic (promotes food intake) in teleosts (Volkoff et al., 2009), the potential role of NPY or indeed the family of NPY hormones in the regulation of food intake in elasmobranchs is largely extrapolated from studies on teleosts and mammals (Demski, 2012). While many of the assumptions in Demski's review are sound they remain assumptions and to date only a single paper has examined the endocrine regulation of feeding in an elasmobranch, *L. ocellata* (MacDonald and Volkoff, 2009). NPY mRNA expression was shown to increase following a 2-week fast (MacDonald and Volkoff, 2009), which is in agreement with previous studies on teleosts (Silverstein et al., 1998; Narnaware and Peter, 2002). However, experimental conditions and species differences are all too common in feeding studies in fish (Hoskins and Volkoff, 2012) and in the same study mRNA expression of CCK increased in the intestine of *L. ocellata* following fasting, whereas similar conditions led to decrease in CCK expression in the winter flounder, *Pseudopleuronectes americanus*, (MacDonald and Volkoff, 2009) and yellowtail, *Seriola quinqueradiata*, (Murashita et al., 2006). The authors suggested this was due to the reduced pH in the intestine of fasting elasmobranchs (MacDonald and Volkoff, 2009), but perhaps more importantly it highlights the need for caution when extrapolating results from teleosts to elasmobranchs.

5. THE HEART AS AN ENDOCRINE GLAND

Definitive evidence of the vertebrate heart as an endocrine organ came with the landmark discovery of De Bold and colleagues in the early 1980s. They reported natriuretic and hypotensive responses from atrial extracts in

the rat (De Bold et al., 1981). Subsequently a family of natriuretic peptides have emerged in the vertebrate lineage with 7 variants being identified, ANP, BNP, VNP, and CNP-1, 2, 3, and 4 (Inoue et al., 2003). Physiological regulation of the renal and cardiovascular systems are hallmarks of natriuretic peptides in vertebrates with typical responses to natriuretic peptide administration being natriuresis (increase in sodium excretion), diuresis (increase in water excretion), and vasodepression (decrease in blood pressure) (Takei, 2000).

In elasmobranchs, CNP is the only natriuretic peptide that has been identified in 4 species of shark; *S. acanthias*, *S. canicula*, *T. scyllia*, and *C. leucas* (Schofield et al., 1991; Suzuki et al., 1991, 1992; Anderson et al., 2005); and 4 species of batoid, the Sawtooth Shark, *Pristis microdon*, *R. typus*, the Banjo Ray, *Trygonorrhina fasciata*, and *D. akajei* (Hyodo et al., 2006). The amino acid sequence of the mature peptide, CNP-22, is identical in all eight species with the exception of two amino acids differing in the sequence for *S. acanthias* (Hyodo et al., 2006). Furthermore, molecular analysis indicates that CNP is the only natriuretic peptide expressed in elasmobranchs (Kawakoshi et al., 2001). With the discovery of CNP in elasmobranch it was suggested that this may be the ancestral natriuretic peptide in vertebrates; however, a natriuretic peptide was subsequently identified in the hagfish (Kawakoshi et al., 2003) and additional data-mining of the Spotted Green Pufferfish, *Tetrodon nigroviridis*, genome revealed 4 distinct CNP genes (Inoue et al., 2003) of which CNP-3 is the likely ancestor to the CNP identified in elasmobranchs (Inoue and Takei, 2006).

The physiological actions of natriuretic peptides are mediated through guanylyl cyclase (GC) coupled natriuretic peptide receptors (NPRs) of which at least three GC-coupled and two GC-non-coupled receptors have been identified in fish (Inoue and Takei, 2006). The GC-coupled receptors include two types of NPR-A and one type of NPR-B identified in the Japanese Medaka, *Oryzias latipes*. The dimeric non-GC-coupled NPR-C receptor is known as a clearance receptor and is thought to bind the natriuretic peptide and internalize it into the cell for degradation. The NPR-D receptor identified in the Japanese eel, *Anguilla japonica*, is also not coupled to a GC but the tetrameric configuration of the receptor is more akin to NPR-A and NPR-B and it is differentially expressed in tissues of *A. japonica*, implying regulation of function (Inoue and Takei, 2006). In elasmobranchs, mRNA expression of an NPR-B type receptor was found in the rectal gland, kidney, gill, and interrenal gland of *S. acanthias*, *T. scyllia*, and *D. sabina* (Sakaguchi and Takei, 1998; Aller et al., 1999; Evans et al., 2010a); all tissues that are involved with regulating volume and salt content of the body fluids and, therefore, consistent with the physiological role of natriuretic peptides in vertebrates. Furthermore, binding studies have

revealed two types of receptor, NPR-B and NPR-C (although this may be an NPR-D) on the gills of *T. scyllia* and gills and rectal gland of *S. acanthias* (Gunning et al., 1993; Donald et al., 1997; Sakaguchi and Takei, 1998), which suggests a role for NPR-C to act as a clearance receptor and therefore regulating circulating levels of CNP.

Administration of CNP to elasmobranchs either *in vitro* or *in vivo* has resulted in vasodilation or decrease in blood pressure respectively (Bjening et al., 1992; Evans et al., 1993). In *S. acanthias*, administration of homologous CNP *in vivo* produced a biphasic response in blood pressure where a transient pressor response was followed by a prolonged depressor response. Coincidental with the pressor response was a 15-fold increase in circulating levels of noradrenaline (McKendry et al., 1999), which suggested CNP may stimulate catecholamine release from the axillary bodies. However, *in situ* perfusion of a cardinal sinus preparation of *S. acanthias* had no effect on catecholamine release from axillary bodies but it was later determined that the NP-stimulated increase in noradrenaline was in some way mediated through the adrenergic system (Monpetit et al., 2001).

As a natriuretic peptide one might intuitively look to the regulation of rectal gland and renal function as a physiological role for CNP in elasmobranchs. The stimulatory actions of natriuretic peptides on rectal gland function were first described in *S. acanthias* using mammalian atriopeptin (Solomon et al., 1985) and later confirmed using homologous CNP (Solomon et al., 1992). We now know that the actions of CNP are mediated through a NPR-B guanylyl coupled receptor (Gunning et al., 1997) and augment the stimulatory actions of VIP. The intracellular signaling pathway for CNP initiated chloride secretion by rectal gland epithelial cells was recently described and involves CNP binding to the basolateral membrane, production of cGMP, and ultimately excretion of chloride via the cystic fibrosis transmembrane regulator (CFTR) chloride channel on the apical membrane via phosphodiesterase, cyclic AMP, and protein kinase A (De Jonge et al., 2014). The significance of blood flow on rectal gland function was demonstrated in the perfused rectal gland of *S. canicula* (Shuttleworth and Thompson, 1986) and in the same species perfusion with homologous CNP resulted in a 30% increase in the secretory parenchyma being perfused (Anderson et al., 2002b), but there was no effect on vascular resistance (Anderson et al., 2001a). The observed increase in perfusion was likely the result of vasodilation by CNP and should, at least in part, be one reason for the increase in secretion from CNP treated glands (Anderson et al., 2002b). One final mechanism of rectal gland function was demonstrated histologically with the presence of a thin circular layer of smooth muscle surrounding the rectal gland in three species of elasmobranch, *S. acanthias*, *D. sabina* and the Great White Shark,

Carcharodon carcharius, that was responsive to a number of humoral agents, suggesting that it may be involved in regulating the intermittent secretory nature of the rectal gland, that is, when the smooth muscle contracts the rectal gland would secrete fluid (Evans and Piermarini, 2001). It appears therefore that control of rectal gland secretory activity may involve a complex integration of cellular regulation of ion transport, vascular constriction, or dilation of the secretory parenchyma and smooth muscle contraction/relaxation surrounding the entire gland.

In regard to renal function, *in vivo* administration of heterologous ANP to *S. acanthias* resulted in a significant decrease in blood pressure followed by a significant antinatriuresis and antidiuresis (Benyajati and Yokota, 1990); but *in situ* perfusion of the kidney in *S. canicula* with CNP resulted in a significant diuresis and natriuresis (Wells et al., 2006). The latter study is more consistent with the salt excreting role of natriuretic peptides in vertebrates. In the study conducted by Benyajati and Yokota (1990), vascular effects were completed within 2 h of natriuretic peptide administration but renal effects were not significant until 3 h following natriuretic peptide administration. It is possible therefore that the *in vivo* results reported by Benyajati and Yokota (1990) were due to the stimulation of local antinatriuretic and vasopressor factors such as angiotensin II within the kidney (Wells et al., 2003) that were released in response to the initial decrease in blood pressure, thus masking the natriuretic and diuretic effects of CNP. Previously it was indicated that support for a tubular action of AVT in the elasmobranch kidney was weak (Wells et al., 2002) and similar observations for CNP have been made. In the *in situ* perfused kidney of *S. canicula*, renal clearance of urea, sodium, and chloride were all significantly increased following administration of CNP; however, relative clearance of these osmolytes was unaffected suggesting that the change in clearance was principally due to a vascular effect of CNP increasing glomerular filtration rate with little if any effect on tubular transport processes (Wells et al., 2006).

In teleosts, atrial stretch as the result of volume loading has been shown to be a powerful stimulus for the release of natriuretic peptides from the heart (Cousins and Farrell, 1996), indeed it has been suggested that release of natriuretic peptides in response to volume loading plays a key role in protecting the heart from overstretching (Farrell and Olson, 2000). Experiments using donor fish that were volume loaded demonstrated the release of a humoral agent that stimulated rectal gland function in *S. acanthias* (Solomon et al., 1984), and led to the hypothesis that volume loading in an elasmobranch would result in an increase in circulating levels of natriuretic peptide that would in turn cause an increase in rectal gland secretion (Silva et al., 1999). In support of the initial findings by Solomon et al. (1984), rectal glands from *S. canicula* acclimated to 70% seawater (volume loaded) had a significantly

greater secretion rate than rectal glands from *S. canicula* acclimated to 120% seawater (volume depleted), furthermore, perfusion of the secretory epithelia was significantly higher in the 70% acclimated fish similar to the pattern observed in rectal glands perfused with CNP (Anderson et al., 2002b).

The euryhaline Bullshark, *C. leucas* migrates between freshwater and seawater as part of its natural life cycle and one might anticipate that *C. leucas* would be volume loaded in freshwater and volume deplete in seawater. Therefore, based on the notion that volume loading causes an increase in plasma CNP, one would anticipate higher plasma concentrations of CNP in freshwater acclimated fish. However, plasma CNP concentration was higher in seawater acclimated *C. leucas* compared to freshwater acclimated fish (Anderson et al., 2005, 2006). To understand this relationship, one needs to consider the salt secreting role of the rectal gland and the volume regulating role of the kidney alongside the life cycle of *C. leucas*. Clearly in freshwater acclimated *C. leucas* there would be a need to retain salt and void water, which would require minimal rectal gland secretion rate and maximal urine flow rate; and in seawater acclimated animals there would be the need to maximize rectal gland secretion and minimize urine flow rate at least in the short term (Hazon et al., 2003; Ballantyne, 2013). While there is no data on renal function in *C. leucas*, adaptation of *S. canicula* to 85% seawater results in a significant increase in glomerular filtration rate and urine flow rate from the isolated perfused kidney (Wells et al., 2002), thus supporting the increase in urine flow from kidneys of elasmobranchs acclimated to reduced salinity. However, in *C. leucas* there was no real difference in key rectal gland measurements from fish acclimated to freshwater or seawater for seventeen days (Pillans et al., 2008) and in long-term seawater and freshwater adapted *D. sabina* NPR-B expression was consistently highest in the rectal gland (Evans et al., 2010a). Combined, these studies suggest that the rectal gland, renal, and natriuretic peptide system in euryhaline elasmobranchs may be in a state of readiness for life in a marine environment, however, the hormonal trigger(s) for activation are as yet unexplained.

Movement between freshwater and seawater environments provides an immense osmoregulatory challenge for fishes and one well-documented physiological response as the fish moves from a low salinity to a high salinity is drinking (Takei and Balment, 2009; Takei and Loretz, 2011). Angiotensin II is recognized as a potent dipsogenic agent (see Section 6) and a drinking response following environmental manipulation and administration of angiotensin has been shown in elasmobranchs (Anderson et al., 2002c). Furthermore, intra-arterial administration of CNP was shown to inhibit Ang II stimulated drinking in *S. canicula* (Anderson et al., 2001c) similar to the effect described of ANP in teleosts (Takei, 2000). It is not known if this

inhibitory effect acts through central regulation of the glossopharyngeal-vagal motor complex as described in teleosts (Ando et al., 2003), but is consistent with the volume regulating role of CNP in elasmobranchs.

6. THE KIDNEY AS AN ENDOCRINE GLAND

In the closed vertebrate circulatory system, regulation of blood volume and blood pressure go hand in hand, and as such the cardiovascular and renal systems are intimately linked. In mammals, a moderate increase in plasma sodium and chloride concentrations leads to an increase in vascular fluid volume that is quickly handled by the kidneys to increase sodium and chloride excretion, and thus bring the fluid volume and salt content back to steady state. One key endocrine system that is involved in this process is the renin angiotensin system, (RAS). Renin produced in granulated cells in the juxta-glomerular apparatus (JGA) of the kidney cleaves the decapeptide angiotensin I (Ang I) from the N-terminus of the precursor polypeptide angiotensinogen. Ang I is further processed by angiotensin converting enzyme (ACE) to form the octapeptide, and principle bioactive component of the RAS, angiotensin II (Ang II) (Fig 8.4). In most vertebrates examined to date Ang II essentially acts

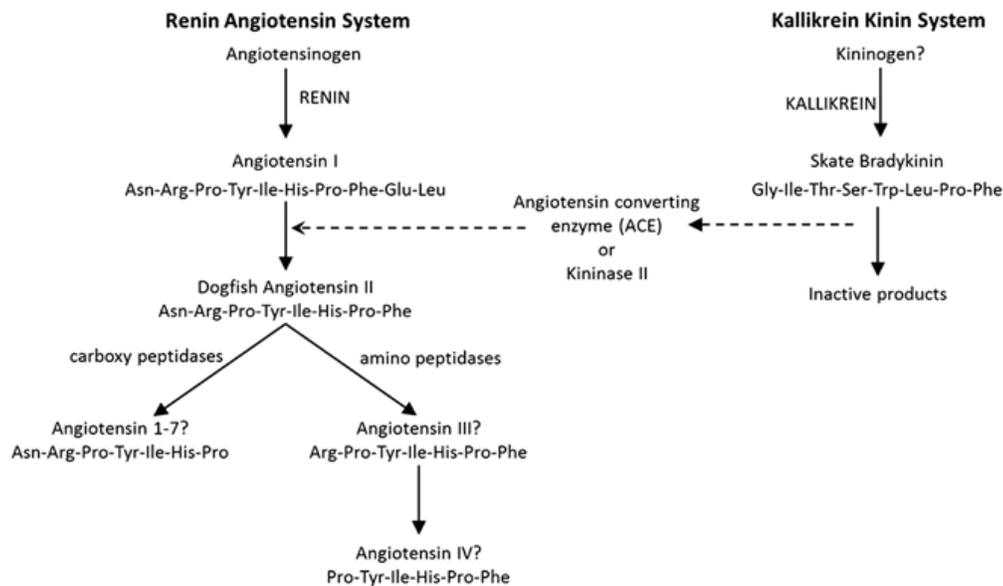


Figure 8.4. Interaction between the renin angiotensin system (RAS) and the kallikrein kinin system (KKS). Note the shared enzyme angiotensin converting enzyme (ACE) or kininase II are the same. Peptides with a question mark have yet to be identified in elasmobranchs. Adapted from Takei and Balment (2009).

in an antagonistic fashion to natriuretic peptides in that it is dipsogenic, vasopressor, antidiuretic and antinatriuretic.

Initial attempts to identify a RAS endogenous to elasmobranchs were not promising. Renal extracts from an elasmobranch failed to induce a pressor response in the rat (Bean, 1942; Nishimura et al., 1970) and there was little evidence to support the presence of granulated JG cells in the kidneys (Capreol and Sutherland, 1968; Nishimura et al., 1970). However, further examination revealed granular peripolar cells and a JG-like structure in the elasmobranch kidney (Lacy and Reale, 1989, 1990). Definitive evidence was provided with the identification of Ang I from *T. scyllia* where it was shown that elasmobranchs have a unique substitution of proline for valine at position three in the amino acid sequence (Takei et al., 1993). Since then angiotensinogen has been identified in three different species of elasmobranch with expression highest in the liver then kidney, interrenal gland, and rectal gland (Watanabe et al., 2009). Binding of radiolabeled Ang II was shown in a variety of tissues from *T. scyllia* and was highest in the interrenal gland (Tierney et al., 1997b). Most recently a specific Ang II receptor was identified in *D. sabina* (Evans et al., 2010a) where it was found to be most similar to mammalian AT₁ type angiotensin receptor as opposed to the AT₂ type. Receptor expression was examined in both freshwater and seawater populations of *D. sabina* and found to be highly expressed in the interrenal gland of both populations then equally expressed in the kidney, gill, and rectal gland of freshwater *D. sabina* and in order of expression level the kidney, gill, and rectal gland of seawater *D. sabina* (Evans et al., 2010a). In support of the AT₁ type classification in elasmobranchs, Ang II stimulated steroidogenesis in interrenal tissue of *S. canicula* required calcium to mediate the response (Armour et al., 1993a), which indicates signal transduction via a calcium signaling pathway, which in mammals is typically associated with the AT₁ receptor (Kaschina and Unger, 2003). Therefore, despite Nishimura's original hypothesis that the evolution of the modern day RAS began with teleosts (Nishimura et al., 1970), it appears that all the requisite components of the RAS exist in elasmobranchs with even the suggestion that individual organs such as the rectal gland (Masini et al., 1994) and kidneys (Wells et al., 2003) may have a local RAS.

A vasopressor effect of Ang II in elasmobranchs was first demonstrated following the *in vivo* administration of heterologous Ang II in *S. acanthias* (Opdyke and Holcombe, 1976). Subsequent pharmacological manipulation of the endogenous RAS in *S. canicula* using the smooth muscle relaxant papaverine, and the ACE inhibitor captopril confirmed the presence of a cascade system analogous to mammals (Hazon et al., 1989); and the homologous peptide was able to induce a dose-dependent vasopressor response in three species of elasmobranch (Takei et al., 1993;

Tierney et al., 1997a; Bernier et al., 1999). In mammals, components of the RAS are known to stimulate catecholamine release from the adrenal medulla (Peach, 1971) and administration of homologous Ang II to *S. acanthias* resulted in a significant increase in plasma concentrations of noradrenaline (Bernier et al., 1999). Furthermore, the vasopressor response in *S. canicula* was mediated through the α -adrenergic system (Bernier et al., 1999) as pre-treatment with the α_2 -adrenoreceptor antagonist yohimbine inhibited the Ang II induced vasopressor response. However, in *S. canicula* the Ang II vasopressor response was not effected by prior treatment with the α -adrenergic antagonist phentolamine either *in vivo* (Tierney et al., 1997a) or *in vitro* (Hamano et al., 1998), which suggests a direct action of Ang II on vascular constriction. Binding of radio-labeled Ang II to gill blood vessels of *T. scyllia* support this notion (Tierney et al., 1997b). Regardless of species differences it is evident that Ang II is involved in the regulation of blood pressure; however, the extent of involvement is likely more concerned with emergency response to decreases in blood pressure rather than constant regulation of minute-to-minute vascular tone.

A major role for the RAS in mammals is the regulation of drinking, where a 1–2% volume loss in the cellular fluid compartment (cellular dehydration) rapidly induces a dipsogenic response that is largely driven by Ang II (Fitzsimons, 1998). In marine elasmobranchs one might not anticipate a drinking response would be present given their osmoregulatory strategy (see Chapter 5); however, drinking has been demonstrated in three species, *S. acanthias*, *T. scyllia*, and *S. canicula* and Ang II has been shown to be a potent dipsogen in *S. canicula* (Hazon et al., 1989). Interestingly, cellular dehydration was without effect in inducing a drinking response in *S. canicula*; however, controlled hemorrhaging of as little as 5.7% blood volume led to a 36-fold increase in drinking (Anderson et al., 2002c), which is consistent with the emergency role of Ang II in blood pressure regulation. Indeed the observed increase in drinking following controlled hemorrhage in *S. canicula* coincided with an increase in circulating levels of Ang II (Anderson et al., 2002c).

The antinatriuretic actions of Ang II in mammals is largely driven by the release of aldosterone from the adrenal gland, which then targets the kidney to enhance sodium reabsorption and potassium excretion. Elasmobranchs do not have aldosterone, but the stimulatory action of Ang II on the release of 1α -OH-B from the isolated perfused interrenal gland has been documented (Armour et al., 1993a; Anderson et al., 2001a; Evans et al., 2010b). Given the apparent sodium regulatory nature of 1α -OH-B in elasmobranchs these data imply a role for Ang II in the regulation of plasma sodium concentration in elasmobranchs. Additional evidence to support this is seen with the antidiuretic effect of Ang II on the elasmobranch kidney

using the *in situ* perfused preparation (Wells et al., 2003, 2006) and the presence of Ang II binding in the rectal gland (Galli and Cook, 1993; Tierney et al., 1997b). In the *in situ* perfused kidney preparation, Ang II significantly decreased perfusion flow rate and caused a significant increase in the population of nonperfused nephrons, but had no effect on the relative clearance of sodium, chloride, or urea (Wells et al., 2006), which indicates that the renal actions of Ang II were predominantly vascular in nature. In the isolated perfused rectal gland, administration of Ang II had no effect on chloride clearance rate or perfusion of the secretory epithelia but binding of radiolabeled Ang II was found to be most abundant in the subcapsular region of the gland between the secretory parenchyma and the surface of the gland (Hazon et al., 1997) and Ang II significantly increased perfusion pressure, which suggests constriction of vessels (Anderson et al., 2001a).

An endocrine system closely associated with the RAS through the shared enzyme, ACE, is the kallikrein kinin system (KKS) (Fig. 8.4). Bradykinin (BK), an active component of the KKS, is involved in the regulation of the cardiovascular system and drinking in teleosts (Olson et al., 1997; Takei et al., 2001). In mammals, the actions of BK are mediated through the B1 and B2 receptors with the preferred ligand for B1 being des-Arg⁹-BK (Bhoola et al., 1992). BK was isolated from heat denatured plasma in *L. erinacea* and found to lack the N-terminal arginine, so is more structurally related to mammalian des-Arg⁹-BK, which suggests that the receptor for BK in elasmobranchs maybe more B1-like (Anderson et al., 2008). Further physiological studies in *L. erinacea* demonstrated a vasopressor action both *in vivo* and *in vitro*. There was a dose-dependent vasopressor response following *in vivo* administration of homologous BK to *L. erinacea* (Dasiewicz et al., 2011). Addition of homologous BK to isolated arterial ring preparations of the mesenteric, coeliac, and branchial artery from *L. erinacea* all resulted in constriction with pharmacological manipulation, suggesting the vasoconstriction may be mediated through the α adrenergic or leukotriene pathways (Dasiewicz et al., 2011).

7. THE PINEAL

The activity of the pineal organ and production of melatonin follows a rhythmical pattern in most vertebrates thus far examined and light is the universal inhibitor of activity. In elasmobranchs, the pineal gland, or organ as in most fishes, is photoreceptive and acts both centrally, through extensive neural connections, and as an endocrine gland through the release of the indoleamine, melatonin (Mandado et al., 2001). There are numerous

cell types within the pineal organ including glial cells, nerve fibers, and photoreceptive cells (Vigh-Teichmann et al., 1983). In many elasmobranchs, the pineal organ is situated in a small window on the dorsal surface of the skull (Gruber et al., 1975) with several lines of evidence indicating photosensitivity. Immunohistochemical studies have shown a strong anti-opsin effect on the surface of the pineal organ in *R. clavata* (Vigh-Teichmann et al., 1983); exposure to light significantly inhibited electrophysiological activity in the pineal stalk of *S. canicula* (Hamasaki and Streck, 1971); and the pineal gland was shown to be involved in the palling response for *S. canicula* during periods of darkness (Wilson and Dodd, 1973a). The response shown in the final study may have been mediated by the release of melatonin from the pineal, which is known to aggregate melanin in melanophores (Gern and Karn, 1983), or through the inhibition of MSH release from melanotropes in the NIL. In support of the latter hypothesis, binding sites for melatonin have been identified throughout the brain of *L. erinacea* (Vernadakis et al., 1998). However, the relative importance of central regulation by melatonin versus secretion and humoral actions of the hormone in elasmobranchs is unknown. The physiological roles of melatonin are quite varied in vertebrates and include regulation of circadian rhythms, seasonal breeding, and color change (Gern and Karn, 1983). In the luminescing sharks, melatonin induces aggregation of pigment in melanophores, thus, uncovering photophores, allowing an increase in light production (Claes et al., 2012). A role for melatonin in regulation of seasonal breeding cycles in elasmobranchs is not well established; however, neural connections between the pineal organ and gonadotrophin releasing hormone immunoreactive nucleus suggest a possible regulatory role for melatonin in secondary pineal circuits (Mandado et al., 2001), one of which may be the hypothalamic pituitary gonadal axis.

8. CALCIUM REGULATION

Appropriate regulation of extracellular fluid calcium is critical to maintaining normal physiological function in all vertebrates. In mammals, parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D work as hypercalcemic hormones, whereas calcitonin (CT) and stanniocalcin (STC) work as hypocalcemic hormones. One might think that the need for hypercalcemic hormones in fish that are surrounded by a ready supply of ionic calcium is somewhat redundant. However, a closely related hormone, PTH-related peptide (PTHrP) has been identified in teleosts and PTHrP immunoreactivity has been shown in a number of elasmobranchs (Trivett et al., 1999, 2001, 2002). Using a heterologous antibody and molecular probe

immunoreactive PTHrP and mRNA PTHrP expression was identified in numerous tissues including the gills, kidney, and rectal gland of elasmobranchs (Trivett et al., 2002), which suggests a potential osmoregulatory role for the peptide, perhaps as a hypercalcemic factor as postulated in the sea bream, *Sparus aurata* (Guerreiro et al., 2001). However, transfer of *S. canicula* to reduced environmental salinity had no effect on tissue expression or circulating levels of PTHrP (Trivett et al., 2001), although the authors suggest that the sampling point of 7 days post-transfer was likely too long to see any effect on circulating levels of PTHrP. It is important to note that all available information on PTHrP is based on the use of heterologous antibodies as the native hormone has yet to be identified in elasmobranchs.

Calcitonin is a well-recognized hypocalcemic hormone that acts antagonistically to the action of PTH and PTHrP in mammals. It is secreted from C-cells in the mammalian thyroid gland and from parenchymal cells in the ultimobranchial bodies (UBB) of non-mammalian vertebrates (Copp et al., 1967). Heterologous bioassays demonstrated strong hypocalcemic activity of *S. suckleyi* UBB in the rat (Copp et al., 1967), which was confirmed following the synthesis of calcitonin from *D. akajei* (Takei et al., 1991). The physiological actions of calcitonin are somewhat more puzzling in elasmobranchs, the lack of a bony skeleton (and thus internal calcium stores) is suggestive of alternate regulatory routes for this hormone in regard to calcium regulation, but results are equivocal. *In vivo* administration of salmon calcitonin to *S. acanthias* had no effect on renal function (Hayslett et al., 1972) and administration of salmon calcitonin to the Leopard Shark, *Triakis semifasciata*, resulted in a marked hypercalcemic response (Glowacki et al., 1985), but administration of salmon calcitonin to the ray, *D. akajei*, resulted in marked hypocalcaemia and hyperphosphatemia (Srivastav et al., 1998). Furthermore, removal of the ultimobranchial body in *D. akajei* resulted in a significant decrease in plasma calcitonin with no effect on plasma calcium levels (Suzuki et al., 1995). A potential role for calcitonin during development in the viviparous *S. tiburo* has been proposed (Nichols et al., 2003). During the yolk dependent stage of development when *de novo* synthesis of calcitonin from the UBB was not possible, increases in maternal calcitonin were coincidental with increases in immunoreactive calcitonin in the duodenum and pancreas of the developing embryo. The authors suggest that the maternal calcitonin may be required during the yolk dependent stage of development; however, immunoreactive calcitonin was not present in the developing gut of *D. sabina* at a similar stage indicating a distinct species difference (Gelsleichter, 2004).

Calcitonin belongs to a family of peptides that includes calcitonin gene related peptide (CGRP) and CGRP has been detected in the brain of

S. canicula (Molist et al., 1995). The receptors calcitonin receptor (CR) and calcitonin receptor-like-receptor (CRLR) were recently cloned from the euryhaline stingray, *D. akajei* (Suzuki et al., 2012). Interestingly expression of both receptors decreased in the gill and kidney following transfer of the stingray from 100% seawater to 20% seawater suggesting that, calcitonin through the associated receptors, may be involved in osmoregulation in the stingray.

Reports of a physiological role for vitamin D and staniocalcin in elasmobranchs are scant in the literature. It is known that vitamin D is present in elasmobranchs, likely from dietary uptake (Sunita Rao and Raghuramulu, 1999); however, a physiological function has yet to be described and levels were undetectable ($<5 \text{ ng ml}^{-1}$) in a survey of three species of wild caught elasmobranchs (Haman et al., 2012). A staniocalcin-like hormone has been detected in elasmobranchs (Wagner et al., 1995) and extracts of goldfish corpuscles of Stannius, the site of synthesis of staniocalcin in teleosts, induced acute, but transient hypocalcemia and hyperphosphatemia in *D. ajakei* (Srivastav et al., 1996). The mechanism of action of staniocalcin in *D. ajakei* is unknown but it does indicate a potential role for this hormone in elasmobranchs despite the lack of anatomically distinct corpuscles of Stannius in this group of fishes.

9. CONCLUSIONS AND PERSPECTIVES

There are approximately eight hundred and fifteen recognized species of elasmobranchs of which three hundred and fifty nine are sharks and four hundred and fifty six are rays (Nelson, 2006). Much of the research discussed in this chapter has, for the simple reason of convenience and abundance of the species, focused on two species of shark, *S. canicula* and *S. acanthias*, and a handful of rays, in particular *L. erinacea* and *L. ocellata*. Thus, it is fair to say that our understanding of elasmobranch endocrinology is based on a small fraction of the total number of species. Given the varied ecology and life history strategies that this highly evolved group of fishes displays, it is reasonable to assume that significant differences in endocrinology have also arisen over evolutionary time; an argument that is abundantly clear with the interspecies differences in catecholamine response to hypoxia, angiotensin mediated effects on blood pressure and VIP stimulation of rectal gland secretion observed between *S. canicula* and *S. acanthias*. While elements of the classic vertebrate hypothalamic-pituitary endocrine axes have been identified, key components remain unidentified. The unique nature of the elasmobranch corticosteroid,

1 α -OH-B, has resulted in numerous tried but failed synthesis attempts, yet the steroid is desperately needed to determine its role in the elasmobranch stress response. The complexity of the elasmobranch nephron makes determining endocrine regulation of renal function particularly challenging. Interest in the evolutionary history of gastro-entero-pancreatic hormones has resulted in molecular identification of many GEP peptide hormones, which led to synthesis of homologous peptide hormones for ultimate use in physiological studies. The rectal gland has served, and will continue to serve, as a key tissue in elasmobranch research providing much information on the cellular basis of sodium and chloride transport and the hormonal control linking gut function and salt balance. Elasmobranchs will remain a challenging model for endocrine and physiological studies, and will continue to act as pivotal components in our understanding of the evolutionary underpinnings of endocrine systems in vertebrates.

ACKNOWLEDGMENTS

The author thanks two anonymous reviewers for their constructive comments on this chapter. I am also grateful to all my mentors and colleagues past, present, and future for helping to formulate many of the ideas presented in this chapter. In particular I thank Drs. Hazon, Takei and Conlon for their invaluable guidance during my early years.

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Physiology of Elasmobranch Fishes: Internal Processes, Volume 34B of the *Fish Physiology* series, together with **Physiology of Elasmobranch Fishes: Structure and Interaction with Environment, Volume 34A**, presents a broad exploration of our current knowledge of elasmobranch physiology. New scientific investigations on elasmobranchs have shed light on their evolutionary history and importance as representatives of ancestral vertebrates, and sophisticated laboratory and field studies have generated a large pool of previously unassembled data.

Both of the volumes provide a comparative presentation of elasmobranch physiology, featuring authors with appropriate expertise and active research programs in the field. Each synthesizes the available data into a comprehensive review of the topic, while also comparing elasmobranchs to other groups of fishes and providing a perspective on future research problems and directions. The current state of knowledge of the elasmobranchs is surprisingly extensive, and these volumes bring to the reader an exhaustively referenced and up-to-date synthesis of the physiology of these fascinating animals.

Key Features

- Coverage extends from molecular and cellular processes to field measurements of whole organismal responses to environmental factors
- Chapters include suggestions for future research and many draw attention to the potential physiological challenges faced with future climate change
- All chapters are written by researchers who are internationally recognized experts in their fields

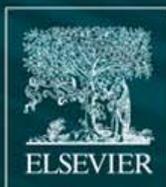
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Cover image: (Top) Blue shark (*Prionace glauca*). Photo taken at the closing light, north western Atlantic. ISO~640, speed~200, aperture ~9. © Joe Romeiro, 333 Productions. (Bottom) Left: Diagram of catshark head showing details of the cranial nerves innervating the respiratory system. Modified from figure 3 in chapter 2, volume 34B. Right: Gill rakers in a megamouth shark (*Megachasma pelagios*). Modified from figure 21 in chapter 3, volume 34A.



ACADEMIC PRESS

An imprint of Elsevier
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ISBN 978-0-12-801286-4



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