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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 O_2 uptake, transport, and delivery, as well as CO_2 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 O_2 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

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plasma accessible CA greatly reduces the interaction between O_2 and CO_2 exchange relative to the situation in teleosts. Moreover, at least in the dogfish, *Squalus suckleyi,* current models of CO_2 excretion suggest similar contributions of the plasma and red blood cell (RBC) to CO_2 excretion, a model that contrasts with the pattern of CO_2 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 HCO_3^- dehydration in the plasma, while HCO_3^- 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 (O_2) and the elimination of metabolic carbon dioxide (CO_2) 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 O_2 delivery and accelerate CO_2 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 portusjacksoni, 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 O_2 and CO_2 transport in the blood.

Table 3.1
Elasmobranchs species commonly discussed in this review

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

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

[a]The Pacific spotted spiny dogfish, formerly considered *Squaluas acanthias*, has been reclassified as *Squalus suckleyi* based on life history and genetic differences (Ebert et al., 2010; Veríssimo 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-O_2 transport characteristics of elasmobranchs were covered in a previous volume of the *Fish Physiology* series (Brauner and Randall, 1998; Gallaugher 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 O_2 across the gills into the blood of elasmobranchs is reviewed by Wegner (2015). The majority of O_2 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 O_2 (PO_2) in the blood is higher than the PO_2 of the metabolically active tissues owing to the steady consumption of O_2, and this difference provides the driving force for O_2 diffusion from the blood into the tissues. Hb-O_2 transport can be "fine-tuned" in response to environmental and metabolic demands by interspecific and intraspecific increases in Hb concentration or through modulating Hb-O_2 binding characteristics (Wells, 1999). Thus, the characteristics of Hb and its microenvironment within the RBCs dictate the nature of blood-O_2 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 O_2 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 O_2 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-O_2 affinity according to environmental and metabolic demands (Hall and McCutcheon, 1938; Bonaventura et al., 1974a;

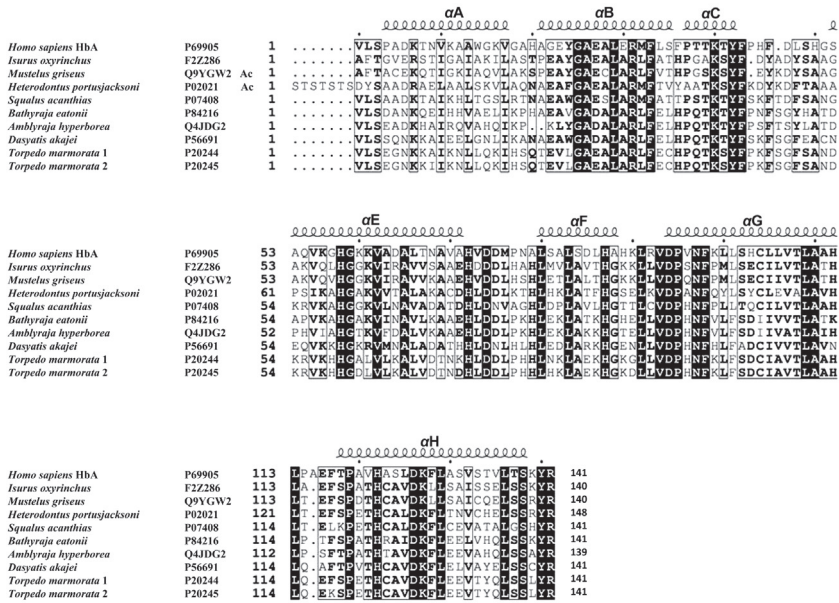
Pennelly et al., 1975; Dickinson and Gibson, 1981; Brittain et al., 1982; Gibson and Carey, 1982; Weber et al., 1983a; Wells et al., 1992; Wells, 1999). Although elasmobranch Hbs do not exhibit the extreme pH sensitivity that is characteristic of teleosts (Pennelly et al., 1975; Farmer et al., 1979; 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 O_2 molecule; thus, one Hb tetramer can bind up to four O_2 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 F8β = His 92β in HbA).

The oxygen equilibrium curve characterizes the relationship between Hb-O_2 saturation and blood PO_2, and the shape and position of the curve reflect Hb-O_2 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-O_2 binding affinity between the sites of O_2 uptake

(A) α-chain



(B) β-chain

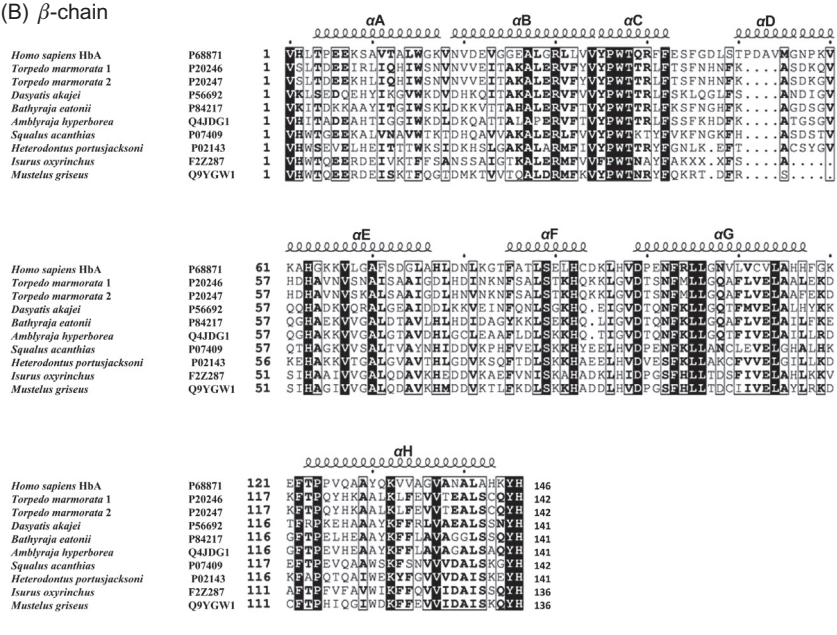


Figure 3.1 (Continued)

and release. The T→R transition is fundamental to the cooperative nature of Hb-O_2 binding, and underlies the sigmoidal shape of the oxygen equilibrium curve because Hb-O_2 affinity increases as successive O_2 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-O_2 affinity is P_{50}, which is the blood PO_2 that corresponds to 50% Hb-O_2 saturation. The evolution of allosteric modulation of Hb-O_2 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 O_2 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								
Dasyatis akajei	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			
Dasyatis americana	Hemolysate	25.5	7.4	15.0	1.7		0.033 M phosphate buffer	McCutcheon (1947)
Dasyatis centroura	Hemolysate	25.5	7.4	15.0	1.6		0.033 M phosphate buffer	McCutcheon (1947)
Dasyatis sabina	Hemolysate	25.5	7.4	15.3	1.6		0.033 M phosphate buffer	McCutcheon (1947)
Dasyatis say	Hemolysate	25.5	7.4	13.5−14.5	1.5		0.033 M phosphate buffer	McCutcheon (1947)
Gymnura micrura (=*Pteroplatea micrura*)	Hemolysate	25.5	7.4	13.0−14.5	1.6		0.033 M phosphate buffer	McCutcheon (1947)
Potamotrygon sp.	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				
Rhinoptera bonasus (=*R. quadriloba*)	Hemolysate	25.5	7.4	14.0	1.2		0.033 M phosphate buffer, pH 7.4	McCutcheon(1947)

Rajiformes								
Amblyraja hyperborea (= *Raja hyperborea*)	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				
Bathyraja eatonii	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				
Raja eglanteria	Hemolysate	25	6.8	37–45			0.05 M phosphate buffer	Hall and McCutcheon (1938)
		25	7.1	38				
		25	7.4	26				
Torpediniformes								
Torpedo nobiliana	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								
Carcharhinus leucas	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
Carcharhinus plumbeus	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				
Galeorhinus galeus (=*G. australis*)	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				
	+1.86 mM IMP		7.3	7.3				
Mustelus canis	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			

Mustelus griseus	Stripped hemolystae	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			
	+2 mM ATP	25	8.5	4.0	2.1			
Mustelus mustelus	Hemolysate	25.5	7.4	7.5	1.3		0.033 M phosphate buffer	McCutcheon (1947)
Negaprion brevirostris (=*Hypoprion brevirostris*)	Hemolysate	25.5	7.4	7.6	1.5		0.033 M phosphate buffer	McCutcheon (1947)
Sphyrna tiburo	Hemolysate	25.5	7.4	7.0	1.2		0.033 M phosphate buffer, pH 7.4	McCutcheon (1947)
Triakis scyllium (=*T. scyllia*)	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			Kono and Hashimoto (1977)
	+GTP	20	6.8	9.7[a]	1.1			
Hexanchiformes								
Notorynchus cepedianus	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								
Lamna nasus	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		$[ATP]/[Hb_4] > 30$	
	+ATP	26		7.4	2.1		$[ATP]/[Hb_4] > 30$	

(*Continued*)

Table 3.2 (Continued)

Species	Sample	°C	pH	P_{50}	n_H	Φ $(\frac{\Delta \log P_{50}}{\Delta pH})$	Buffer/Notes	References
	IsoHb III	10		0.9	1.8	+ 0.5	Φ from pH 7.5 to 8.3	
						−0.6	Φ pH <7.5	
		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								
Squalus acanthias	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)
Squalus suckleyi	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 ($\Delta \log P_{50}/\Delta pH$). °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.

[a]Indicates respective parameter was estimated from data or figure in reference.

Table 3.3
Whole-blood characteristics of chondrichthyan fishes.

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
HOLOCEPHALI														
Chimaeriformes														
Chimaera monstrosa					2.7	15.7	170							Larsson et al. (1976)
Hydrolagus colliei					2.9–3.4			16	1.1[a]	Absent			P_{CO_2} 2.5–28 mmHg, 11°C	Hanson (1967)
ELASMOBRANCHII														
Batoidea														
Myliobatiformes														
Dasyatis guttata					8.2	21.7	389							Filho et al. (1992b)
Dasyatis sabina	90	14.2				14.6							23°C, cannulated	Cameron et al. (1971)
					3.6	24.3								Dabruzzi and Bennett, 2014
Dasyatis say					3.6	14.3	235							Filho et al. (1992b)
Myliobatis californica	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
Myliobatis goodie					4.9	18.0	298							Filho et al. (1992b)
Potamotrygon motoro					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)
Potamotrygon motoro (=*P. circularis*)					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)
(=*P. laticeps*)					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)
Potamotrygon sp.						24		12.3		Present			No CO_2, pH 7.7, 30°C	Martin et al. (1979)
								17.0					P_{CO_2} 42.6 mmHg, 30°C	Martin et al. (1979)
Potamotrygon spp.								11.8−12.7 16.6−19.7		−0.19− −0.26			No CO_2, 30°C P_{CO_2} 43 mmHg, 30°C	Powers et al. (1979b)
Rhinoptera bonasus										−0.41			Washed RBCs, Φ calculated from pH 6.9-8.0, 20°C	Scholnick and Mangum (1991)
Rhinoptera bonasus					6.7	25.1	286							Filho et al. (1992b)
Taeniura lymna					4.8	14.8	318							Baldwin and Wells (1990)
Trygonoptera testacea					3.7	21	173						19°C	Cooper and Morris (1998)
Rajiformes														
Amblyraja radiate (=*Raja radiata*)					3.8	16.7	216							Larsson et al. (1976)

Atlantoraja castelnaui (=*Raja castelnaui*)					2.6	15.4	205							Filho et al. (1992b)
Atlantoraja cyclophora (=*Raja cyclophora*)					5.9	17.9	366							Filho et al. (1992b)
Atlantoraja platana (=*Raja platana*)					4.1	16.3	234							Filho et al. (1992a)
Aptychotrema rostrata	82		1.9		3.0[b]	12.2	242[b]	47.6	2.1		7.8		28°C, P_{50} *in vivo*, cannulated, respirometer	Speers-Roesch et al. (2012a)
Dipturus batis (=*Raja batis*)					2.9	19.0	154							Larsson et al. (1976)
Leucoraja naevus (=*Raja naevus*)					2.4	23.0	106							Leray (1982)
Leucoraja ocellata (=*Raia oscillata*)	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)
Leucoraja ocellata (=*Raja ocellata*)	100		0.8		2.8	12.5	239	27.6	1.8	−0.29	7.83 pHi 7.4		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			pH 7.40, P_{CO_2} 7.50 mmHg, 12°C	
Glaucostegus typus (=*Rhinobatos batillum*)					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	
Glaucostegus typus (=*Rhinobatos typus*)					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
Raja clavata (=*Raia clavata*)	58							30.2	2.5	−0.25	7.7		pH 7.7, 15°C, cannulated	Hughes and Wood (1974)
Raja microocellata					2.9	21.1	113							Leray (1982)
Rajella lintea (=*Raja lintea*)					3.4	19.3	172							Larsson et al. (1976)
Rhinobatos horkelii					6.1	18.9	260							Filho et al. (1992b)
Rhinobatos percellens					4.4	13.3	329							Filho et al. (1992b)
Rioraja agassizii (=*Raja agassizi*)					4.5	17.7	231							Filho et al. (1992b)
Sympterygia acuta					4.7	21.6	226							Filho et al. (1992b)
Sympterygia bonapartii (=*S. bonapartei*)					3.2	13.8	241							Filho et al. (1992b)
Torpediniformes														
Narcine brasiliensis					4.0	17.1	227							Filho et al. (1992b)
Torpedo marmorata	70							20.2		−0.32	7.82		pH 7.8, 15°C, cannulated, respirometer	Hughes (1978)
								28[a]					20°C	
Zapteryx brevirostris					4.9	19.0	247							Filho et al. (1992b)
Selachimorpha														
Carcharhiniformes														
Carcharhinus brevipinna (=*C. maculipinnis*)					7.2	30.1	265							Filho et al. (1992b)
Carcharhinus limbatus					8.4	22.3	278							Filho et al. (1992b)
Carcharhinus melanopterus					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	

Carcharhinus obscurus			6.2	18.2	345							Emery (1986)
			4.8	15.0	324							Filho et al. (1992b)
Carcharhinus plumbeus			5.1	14.9	350							Emery (1986)
Carcharhinus plumbeus (=*C. milberti*)			4.4	16.1	311							Filho et al. (1992b)
			4.01	17.7	228	20.3	2.4	−0.56			pH 7.92, P_{CO_2} 1.5 mmHg, 25°C	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	
Carcharhinus porosus			5.8	29.9	248							Filho et al. (1992b)
Cephaloscyllium isabellum (=*C. isabella*)			3.5[b]	16.8	209[b]	4.83		−0.49			pH 7.674,5°C	Tetens and Wells (1984)
			3.0[b]	15.0	208[b]	8.3	1.5	−0.32			pH 7.7, 15°C	
Cephaloscyllium ventriosum		0.5 – 14.5	2.7	13.5						7.60−8.04	15°C, caudal venepuncture	King (1995)
Galeocerdo cuvier			6.5	19.8	338							Emery (1986)
								−0.38			Washed RBCs, Φ calculated from ~ pH 6.9–7.9, 20°C	Scholnick and Mangum (1991)
Mustelus fasciatus			4.9	23.5	222							Filho et al. (1992b)
Mustelus schmitti			4.2	20.4	221							Filho et al. (1992b)
Negaprion acutidens			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	Wells et al. (1992)
						12.3	2.0	−0.24			Washed RBCs, pH 7.4, no CO_2, 25°C	
Negaprion brevirostris	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)
Prionace glauca			5.0	15.2	332							Emery (1986)
			2.9	11	264						Exercise stressed	Wells et al. (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
Prionace glauca (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)
Scyliorhinus canicula					5.62	21.8		21.5	1.7	−0.43			pH 7.58, P_{CO_2} 2.2 mmHg, 17°C	Pleschka et al. (1970)
								25.9					pH 7.38, P_{CO_2} 7.3 mmHg, 17°C	
Scyliorhinus canicula	90.9	21.3									7.88	7.83	7°C, PiO_2 140 mmHg, cannulated, restrained	Butler and Taylor (1975)
	114.4	34.5									7.81	7.77	12°C, PiO_2 140 mmHg	
	97.6	32.9									7.74	7.68	17°C, PiO_2 131 mmHg	
	13.8	6.6									7.91	7.84	7°C, PiO_2 43 mmHg	
	16.5	7.0									7.74	7.66	12°C, PiO_2 42 mmHg	
	16.8	6.0									7.68	7.64	17°C, PiO_2 39 mmHg	
	95	23									7.76	7.71	15°C, PiO_2 148 mmHg, cannulated, respirometer	Short et al. (1979)
	31	11									7.68	7.59	PiO_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 pHi7.3		15°C, cannulated, flow-through chambers	Wood et al. (1994)
	93		1.1		4[b]	16.7					7.78 pHi7.3		15°C, cannulated, flow-through chambers	Perry et al. (1996)

Scyliorhinus stellaris	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							7.78	7.71	16°C, cannulated, free-swimming	Baumgarten-Schumann and Piiper (1968)
								16	1.8				P_{CO_2} 1.5 mmHg, 17°C, *in vivo* and *in vitro*	Piiper and Baumgarten-Schumann (1968)
	64		2.1										17.8–19.2°C, cannulated, resting	Piiper et al. (1977)
Sphyrna lewini					10.0	26.5	370							Emery (1986)
					8.4	27.3	343							Filho et al. (1992b)
Sphyrna tiburo						19.9–22.2							28°C	Carlson and Parsons (2003)
Sphyrna zygaena					6.6	25.4	281							Filho et al. (1992b)
Triakis semifasciata	66	12	1.8	2.55		18.3		15.3	1.1–1.5		7.78	7.75	P_{50} and n_H *in vivo* at 15°C, blood gases and pH at 19–22°C, cannulated	Lai et al. (1990)
Heterodontiformes														
Heterodontus portusjacksoni	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														
Alopias vulpinus					13.6	37.4	360							Emery (1986)
					11.9	33.0	360							Filho et al. (1992b)
Carcharias taurus (=*Odontaspis taurus*)					5.9	21.9	283							Filho et al. (1992b)
Carcharodon carcharias					13.5	36.0	379							Emery (1986)
Isurus oxyrinchus					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														
Chiloscyllium punctatum					6.3[c]	20.9[c]	278						24°C	Chapman and Renshaw (2009)
					7.5[d]	27.3[d]								
Hemiscyllium ocellatum					3.64	13.4	272							Baldwin and Wells (1990)
Hemiscyllium ocellatum (=*H. oscellatum*)					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)
					6.2[d]	22.8[d]								
	97.5		1.35		2.9[b]	13.4	219[b]	32.0	1.3		7.87		28°C, P_{50} *in vivo*, cannulated, respirometer	Speers-Roesch et al. (2012a)
Squaliformes														
Etmopterus spinax					3.0	18.9	168							Larsson et al. (1976)
Somniosus microcephalus					3.2	20.5	156							Larsson et al. (1976)
Squalus acanthias					2.9	15.3	188							Larsson et al. (1976)
					3.3	20.9	158							Leray (1982)
	111		1.7								7.85		14–15°C, cannulated, resting	Swenson and Maren (1987)
					2.7[b]	11.6	235[b]	13.2	1.7	−0.28			pH 7.85, P_{CO_2} 2.2 mmHg, 15°C	Wells and Weber (1983)

Squalus cubensis					7.3	31.0	234							Filho et al. (1992b)
Squalus suckleyi	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)
(=*S. acanthias*)					3.0	14.8	208						12°C	Perry and Gilmour (1996)
(=*S. acanthias*)	102		1.3								7.8		11°C, cannulated, flow-through chamber	Richards et al. (2003)
(=*S. acanthias*)	117.4	6.3	1.24	1.6							7.87	7.81	13°C, cannulated, flow-through chamber	Gilmour and Perry (2004)
Squantiniformes														
Squatina argentina					4.3	23.1	246							Filho et al. (1992b)

PaO_2, PvO_2, $PaCO_2$, and $PvCO_2$ 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 ($\Delta \log P_{50}/\Delta pHe$)., and pHa and pHv 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.

[a]Indicates respective parameter was estimated from data or figure in reference.

[b]Converted using a constant for human HbA: 1 g dL^{-1} = 0.1551 mmol Hb_4 L^{-1}

[c]Captive sharks

[d]Wild sharks

hemolysate of the salmon shark, *Lamna ditropis* (=*Lamna ditrotus*), 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 O_2 affinity similar to the whole hemolysate, but lower than the two less predominat 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 O_2 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 O_2 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-O_2 affinities than adult sharks, and fetal isoHbs persist for at least 10 days posthatch in *Cephaloscyllium ventriosum*, which may ensure adequate O_2 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-O_2 affinity (see Section 2.3) should benefit prehatch and embryonic individuals by enhancing O_2 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-O_2 affinity (increases P_{50}) in many vertebrates, permitting a relatively rapid rightward shift of the oxygen equilibrium curve associated with CO_2 production during blood capillary transit. This pH dependency of Hb-O_2 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-O_2 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-O_2 affinity with declining pH at values typically outside the physiological range (below pH $\approx$ 6); the latter is present in some elasmobranchs 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 O_2

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:

$$\Phi = \frac{\Delta \log P_{50}}{\Delta \mathrm{pH}} = \frac{1}{4}\Delta z\mathrm{H}^{+} \tag{3.1}$$

where Φ values are equal to one quarter $\Delta z\mathrm{H}^{+}$ 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 $\Delta z\mathrm{H}^{+}$ 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 1α (NA1), His 122α (H5), His 2β (NA2), Lys 82β (EF6), His 143β (H21), and His 146β (HC3) (Perutz, 1983; Berenbrink, 2006; Mairbäurl and Weber, 2012). Because the pK_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 146β) 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 Asp94β 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					
Ameiurus punctatus	−14.3 (25)	−5.8			Cameron and Kormanik (1982)
A. nebulosus	−15.5 (24)	−5.7	−0.12 mmol/g Hb/pH unit		Szebedinszky and Gilmour (2002)
Anguilla rostrata	−10.1 (20)	−2.7			Hyde et al. (1987)
Catostomus commersoni	−8.8 (28)	−2.3			Wilkes et al. (1981)
Hippoglossoides elassodon	−6.6 (14)	−2.1			Turner et al. (1983)
Katsuwonus pelamis	−8.0 (25)	−3.1		4.1	Tufts and Perry (1998) and Jensen (2001)
	−11.2 (41)	−3.1			Perry et al. (1985)
Oncorhynchus mykiss	−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)
Platichthys stellatus	−7.0 (25)	−2.9			Wood et al. (1982)
Salvelinus fontinalis	−7.5 (35)	−3.3			Packer and Sunkin (1979)
Elasmobranchii					
Leucoraja ocellata	−11.0 (13)	−6.6			Tufts and Perry (1998) and Graham et al. (1990)
Raja clavata	−10				Hughes and Wood (1974)
Raja rhina	−6.11 (13.5)	−2.83			Gilmour et al. (2002)
Myliobatis californica	−14.3 (20) to −16.4 (23)				Hopkins and Cech (1974a)

(*Continued*)

Table 3.4 (Continued)

Species	β Whole blood	β Separated plasma	β Hb	ΔzH^+	References
Mustelus asterias			−11.5	1.1	Berenbrink et al. (2005)
Scyliorhinus stellaris	−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)
Squalus acanthias	10–12 (pH 7.85)		−11.7	1.0	Weber et al. (1983a) and Berenbrink et al. (2005)
Squalus suckleyi	−9.0 (13–26)	−6.5			Tufts and Perry (1998) and Lenfant and Johansen (1966)
Triakis semifasciata	−9.3				Lai et al. (1990)

β Whole blood and β separated plasma refers to buffer values in Slykes (mmol HCO_3^- pH unit^{-1} 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 Hb_4), and increases in the magnitude of the Bohr effect evolved independently in the amniotes and early actinopterygiians, 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 146β and Asp94β 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 146β) 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 146β and Asp 94β 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 94β 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 HC1β (Lys 144β 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 ($\Delta z H^+ = 1.0$) (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 F6β (Leu 91β in HbA) may inhibit salt-bridge formation between AspFG1β 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-O_2 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 O_2 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-O_2 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-O_2 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-O_2 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-O_2 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-O_2 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-O_2 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-O_2 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 H21β (His 143β 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 104β and Ala 135β of human HbA are substituted for two positively charged amino acid residues, lysine (G6β) and arginine (H13β), 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 G6β (Arg 104β), but lack a substitution for a positively charged residue at position H13β (Ala 135β). Additionally, the presence of ATP does not have a substantial effect on Hb-O_2 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-O_2 affinity or had very little influence on Hb-O_2 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-O_2 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-O_2 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 O_2 affinity and the oxygenation-linked binding of ATP to elasmobranch Hbs. The urea-induced increase in Hb-O_2 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-O_2 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-O_2 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-O_2 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 O_2 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-O_2 affinity in *Squalus acanthias* and *Cephaloscyllium isabellum*. Therefore, Hb-O_2 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-O_2 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-O_2 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:

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

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 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 ($\Delta H' = -44$ kJ mol^{-1}) and pH 7.0 ($\Delta H' = -35$ 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, *Bathyraja eatonii* and *Amblyraja 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 *Bathyraja eatonii* Hb, the reverse observed in *Amblyraja 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"

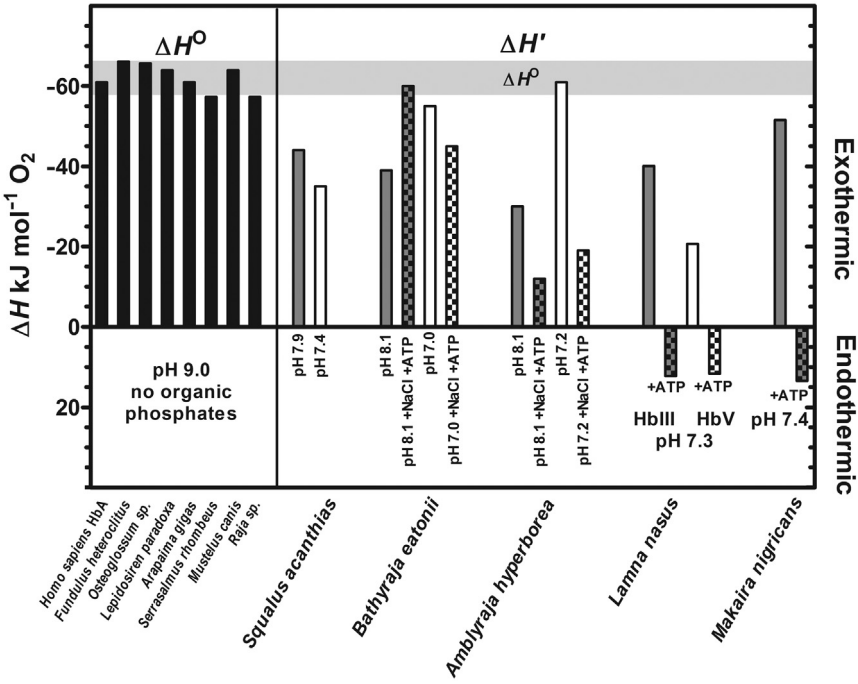


Figure 3.2. Enthalpies of oxygenation (including the heat of solution of O_2, -12.55 kJ mol^{-1} O_2) for stripped hemoglobin from representative jawed vertebrates. Black bars to the left of the vertical line are enthalpies of heme-oxygenation (ΔH^O) measured in the absence of organic phosphates and 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 ($\Delta H' = -40$ kJ mol^{-1}) and IV ($\Delta H' = -21$ kJ mol^{-1}), but reverse in the presence of ATP ($\Delta H' \approx +12$ 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 temperate 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^{O}) 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-O_2 affinity within the RBCs is dependent on the intrinsic O_2 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-O_2 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 O_2 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-O_2 affinity and O_2 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; Gallaugher 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 Gallaugher 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

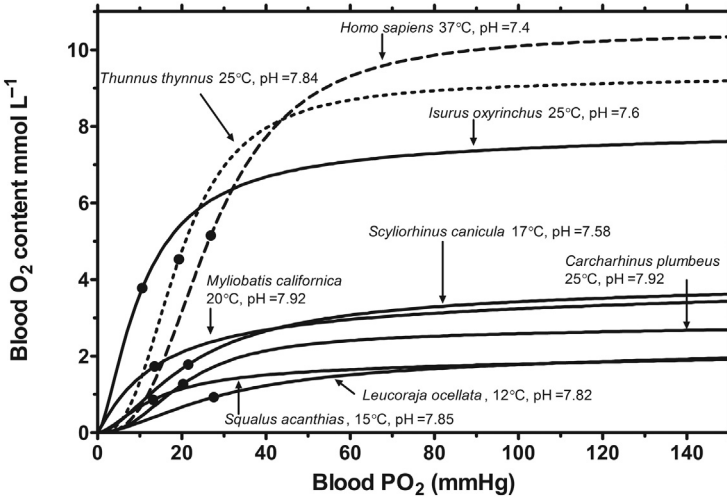


Figure 3.3. Comparison of the relationship between blood-O_2 content (mmol L^{-1}) and blood PO_2 (mmHg) for six elasmobranch species (solid lines), Atlantic bluefin tuna (dotted line; *Thunnus thynnus*), and humans (dashed line; *Homo sapiens*). Blood-O_2 content was determined at corresponding blood PO_2 levels by using Hill's equation, $\alpha bPb + 4[Hb_4]\frac{Pb^n}{P_{50}^n + Pb^n}$ (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), Pb is the blood PO_2, $[Hb_4]$ 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 puntuatum*, 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 puntuatum* 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 O_2-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-O_2 affinity) over the same developmental stages (King, 1994). King (1994) speculated that a high blood O_2-carrying capacity would be more beneficial for free-swimming adult sharks than prehatch 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 PO_2 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; Gallaugher 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 O_2 consumption is complex, vertebrates have evolved a number of different ways to increase O_2 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 O_2-carrying capacity and O_2 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 Gallaugher 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-O_2 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-O_2 affinity) typically is associated with hypoxia-tolerant fishes as it will enhance O_2 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-O_2 affinity) is advantageous for offloading O_2 at high PO_2 in the tissue capillary beds, which will increase O_2 diffusion between the blood and a mitochondrion in the tissues, but can compromise O_2 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 CO_2 (e.g., Graham et al., 1990), although some species exhibit modest Bohr effects *in vitro* (Table 3.3). The CO_2 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_{pHe}) 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 draughtsboard 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 O_2 delivery is likely enhanced by the Root effect that reduces blood O_2-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 O_2 delivery to the tissues.

2.4.2. Exercise

Exercise generally increases the metabolic demand of muscle for O_2, requiring an increase in blood O_2 transport if O_2 supply is to match demand. Transport and supply of O_2 can be increased by increasing blood flow (cardiac output, $\dot{Q}$) or by extracting a greater amount of O_2 from the blood, as described by the Fick principle:

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

where $\dot{M}O_2$ is total O_2 consumption and $CaO_2 - CvO_2$ refers to the arterial–venous O_2 content difference. Whether blood transport of O_2 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 O_2 delivery to the working muscles via the Bohr effect. Increasing the delivery of O_2 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 O_2 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 O_2 supply with demand during acute bouts of exhaustive exercise, owing to a low blood O_2-carrying capacity and the apparent lack of regulated blood-O_2 transport (Piiper et al., 1977; Butler

and Metcalfe, 1988; Lowe et al., 1995; Brill et al., 2008). For example, strenuous exercise ($\leq$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 O_2 content of mixed venous blood was reduced by 55% in spontaneously swimming *Scyliorhinus stellaris* (Piiper et al., 1977). Unexpectedly, venous O_2 stores were unchanged in swimming *Negaprion brevirostris*, but arterial PO_2 (PaO_2) and CaO_2 increased by 40% and 31%, respectively (Bushnell et al., 1982). This increase of arterial blood-O_2 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 PaO_2 and low arterial Hb-O_2 saturation limits branchial O_2 uptake during rest and routine swimming, but allows O_2 loading over a wider PO_2 range in response to demand (Bushnell et al., 1982; Korsmeyer et al., 1997). This strategy of limiting branchial O_2 exchange may have an osmoregulatory or ionoregulatory benefit in the context of the osmorespiratory 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 CO_2 (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 O_2 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 O_2

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 (Piiper 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 O_2-carrying capacity and Hb-O_2 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-O_2 affinity and caused Hct to fall by around 29%, effectively decreasing blood-O_2 content, although blood PO_2, the CaO_2-CvO_2 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 P_{50} 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 Mylibatiform 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

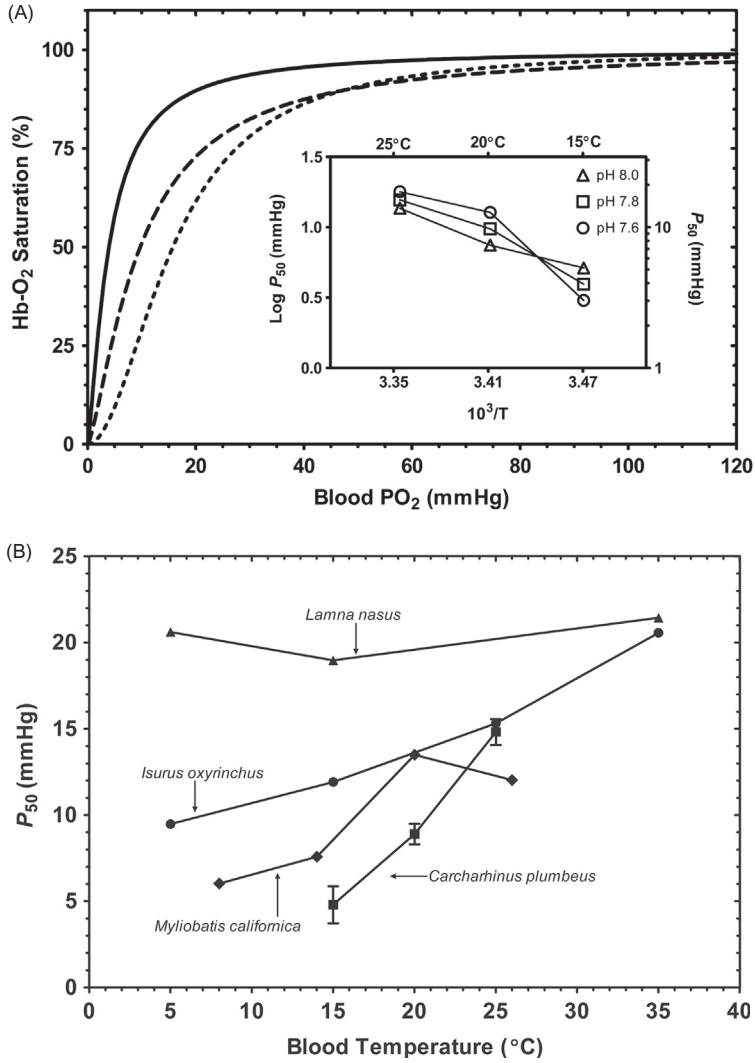


Figure 3.4. The effect of temperature on whole blood Hb-O_2 affinity of elasmobranchs. Oxygen equilibrium curves for *Carcharhinus plumbeus* at pH 7.8 and 15°C (solid line; $P_{50} = 4$), 20°C (dashed line; $P_{50} = 10$), and 25°C (dotted line; $P_{50} = 16$) are shown in panel (A) with an inset of the van't Hoff plot where logP_{50} values were interpolated from logP_{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 though 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-O_2 affinity and blood-O_2 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-O_2 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-O_2 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-O_2 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-O_2 affinity, effectively decreasing O_2 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 O_2 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 CO_2, even though the majority of total CO_2 is carried in the plasma as HCO_3^- 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, CO_2 diffuses into the RBC where it is hydrated to H^+ and HCO_3^- 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 CO_2 loading into the blood. The process is reversed when the blood reaches the gas exchange organ. As molecular CO_2 diffuses out of the RBC and across the gas exchange surface according to its partial pressure gradient, HCO_3^- moves from the plasma into the RBC via band 3 and is dehydrated to CO_2 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 CO_2 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, CO_2 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 CO_2 excretion in the dogfish *Squalus suckleyi*, and potentially elasmobranchs in general, does not follow the typical model of CO_2 excretion in teleosts described above (reviewed by Gilmour and Perry, 2010). The sections that follow will first discuss transport of CO_2 in the blood of elasmobranchs and then focus on CO_2 excretion, with emphasis on the tissue distribution of CA isoforms and their likely roles in CO_2 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 HCO_3^- with smaller amounts transported as physically dissolved CO_2 and as carbamino CO_2 (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), CO_2 transport as carbamino CO_2 bound to Hb may be relatively more important in elasmobranchs than it is in teleosts. The CO_2 combining curve describes the relationship between total CO_2 and blood PCO_2, and thus the CO_2 capacitance of the blood. Arterial and venous PCO_2 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 CO_2 capacitance; for example, CO_2 capacitance in the blood of *Scyliorhinus stellaris* is approximately 50 to 70 times greater than O_2 capacitance (Piiper and Baumgarten-Schumann, 1968). In most elasmobranchs studied to date, the CO_2 capacitance of separated plasma

is similar to that of whole blood and true plasma, whereas separated plasma from teleosts generally has a substantially lower CO_2 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 CO_2 and then centrifuged to yield plasma versus plasma that has been separated from blood and subsequently equilibrated with CO_2 in isolation, and therefore the similarity of CO_2 capacitances among separated plasma, whole blood, and true plasma in elasmobranchs implies significant CO_2 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 CO_2 capacitance exhibited by separated plasma in this shark (Tufts and Perry, 1998); carbamino CO_2 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 CO_2 excretion by providing protons for HCO_3^- dehydration in the plasma (see Section 3.2.3).

In the presence of a Haldane effect, deoxygenated blood contains more CO_2 than oxygenated blood (Christiansen et al., 1914); proton binding to oxylabile sites on Hb enhances CO_2 hydration in the RBC, leading to greater loading of CO_2 into the blood. In elasmobranchs, the Haldane effect generally is considered to be small and to play a negligible role in blood CO_2 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 CO_2 transport in the blood by removing the protons generated by CO_2 hydration in the RBC.

The content and tension of CO_2 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 CO_2 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 CO_2 in plasma decreases as temperature increases (Pleschka and Wittenbrock, 1971), so accordingly at constant total CO_2 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 HCO_3^- 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 HCO_3^- concentrations (Heisler et al., 1980; Heisler, 1988).

Exercise is expected to influence the production of CO_2 and hence blood CO_2 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 CO_2 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 (Piiper 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 HCO_3^- dehydration within the plasma, which facilitates CO_2 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 CO_2 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 Chegwidden 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 CO_2 excretion.

3.2.2. Carbonic Anhydrase Isoforms Involved in CO_2 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 70×10^{-4} 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 cuivieri*, indicates RBC CA exists (Bergenhem 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 CO_2 within gill epithelial cells to provide H^+ and HCO_3^- 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 CO_2 excretion because it is inaccessible to HCO_3^- 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 CO_2 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 Briggle, 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 CO_2 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 CO_2 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 Briggle, 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 CO_2 excretion (Fig. 3.5).

3.2.3. The Elasmobranch Model of CO_2 Excretion

The elasmobranch model of CO_2 excretion posits dual reliance on RBC and plasma dehydration of HCO_3^- as blood passes through the gill. As in the classic model of CO_2 excretion, HCO_3^- carried in the plasma can enter the RBC via the band 3 anion exchanger (Obaid et al., 1979) and be dehydrated to molecular CO_2 in the presence of RBC CA, with protons being provided through the buffering action of Hb. At the same time, however, HCO_3^- ions can be dehydrated to molecular CO_2 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 HCO_3^- dehydration to CO_2 excretion has come from measurements of HCO_3^- 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 HCO_3^- clearance was accompanied by a significant increase in arterial PCO_2 (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, CO_2 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 PCO_2 or HCO_3^- clearance at the gill (Gilmour et al., 1997, 2001). Anion exchange is considered to be the rate-limiting step in HCO_3^-

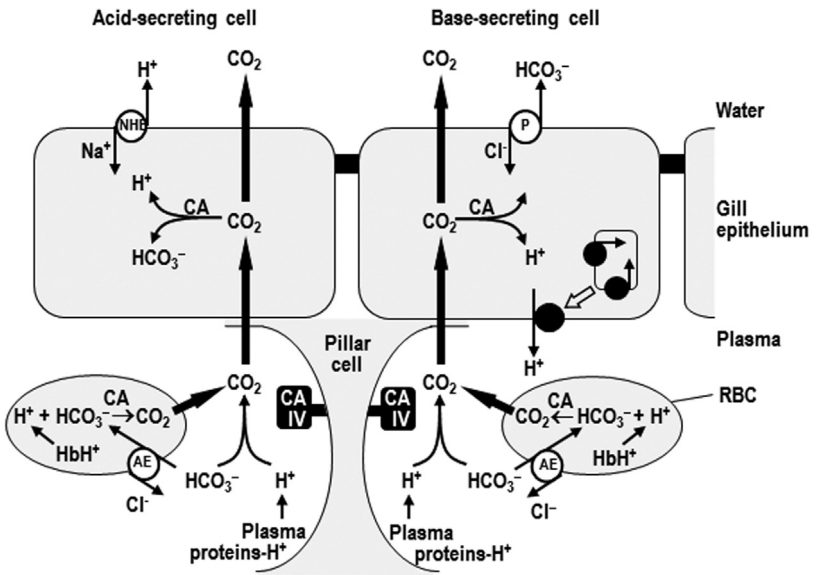


Figure 3.5. A schematic representation of the elasmobranch model of CO_2 excretion, i.e. CO_2 excretion at the gill of *Squalus suckleyi* [Note that the Pacific spiny dogfish, formerly considered Squalus acanthias, has been reclassified as *Squalus suckleyi* (Ebert et al., 2010), but is named Squalus acanthias in much of the literature]. Bicarbonate ions (HCO_3^-) carried in the plasma may enter the RBC for dehydration to CO_2 in the presence of RBC cytosolic CA, or may be dehydrated to CO_2 in the plasma in the presence of pillar cell CA IV. Protons for HCO_3^- dehydration are provided by Hb buffering in the RBC and plasma protein buffering in the plasma. Molecular CO_2 diffuses across the gill epithelium down its partial pressure gradient. Some component of this CO_2 likely is hydrated to HCO_3^- 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 HCO_3^- 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 (Rispens 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 CO_2 Excretion Pathways in Vertebrates

A comparison of CO_2 excretion pathways across vertebrates as a whole yields some insight into the evolution of the elasmobranch model of CO_2 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 CO_2 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 HCO_3^- dehydration can make a substantial contribution to CO_2 excretion (Esbaugh et al., 2009). In agreement with this hypothesis, the majority of the blood total CO_2 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 CO_2 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 HCO_3^- dehydration, collectively resulting in dual reliance on both plasma and RBC (see Section 3.2.3). In both hagfish and elasmobranchs then, O_2 transport and CO_2 transport are effectively uncoupled, with O_2 delivery being dependent upon the RBC and CO_2 excretion exhibiting substantial dependence on the plasma.

In the presence of Hb with a strong Bohr-Haldane effect, O_2 and CO_2 transport can be coupled to benefit both O_2 delivery and CO_2 excretion (reviewed by Brauner and Randall, 1998). Addition of CO_2 to the blood in the tissues lowers Hb-O_2 binding affinity (Bohr effect) to enhance O_2 delivery to the tissues, whereas elimination of CO_2 at the gas exchange organ reverses this effect to the benefit of O_2 loading. As O_2 binds to Hb, it drives off oxylabile protons (Haldane effect) that can then be used for HCO_3^- dehydration to benefit CO_2 excretion, whereas deoxygenation of the blood

at the tissues increases the proton-binding capacity of Hb to the benefit of CO_2 loading into the blood. The large Haldane effect of lamprey Hb is critical for effective CO_2 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 HCO_3^- 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 CO_2 transport in the blood (Tufts and Boutilier, 1989). Proton removal via the pronounced Haldane effect and a secondarily active Na^+/H^+ exchanger allow CO_2 to be loaded into the blood while HCO_3^- ions are retained within the RBC (reviewed by Nikinmaa et al., 1995; Nikinmaa, 1997; Tufts and Perry, 1998). The tight linkage between O_2 and CO_2 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 HCO_3^- 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 CO_2 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 CO_2 hydration/HCO_3^- dehydration reactions with proton production/consumption in close proximity to Hb. It is this pattern of CO_2 excretion that appears to have been largely retained through the tetrapods. Although pulmonary capillary endothelial CA IV is present in tetrapods (Whitney and Briggle, 1982; Zhu and Sly, 1990; Waheed et al., 1992; Stabenau and Heming, 2003), the RBC dominates CO_2 excretion owing to high RBC CA activity and buffer capacity (Henry and Swenson, 2000; Swenson, 2000).

Thus, two basic strategies for CO_2 excretion appear to be present among vertebrates. In one, represented in hagfish and elasmobranchs, the Bohr-Haldane effect is small or absent, O_2 and CO_2 transport are uncoupled, and CO_2 excretion relies to a significant extent on dehydration of HCO_3^- 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 CO_2 hydration/HCO_3^- dehydration reactions; CO_2 excretion is tied to the RBC by coupling it to O_2 uptake. However, with only a handful of species having been studied in any detail, such inferences about patterns of CO_2 excretion and the evolution of CO_2 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 O_2 transport and CO_2 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 O_2 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 O_2, 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-O_2 affinity causes the O_2 capacitance of the blood ($\Delta CO_2/\Delta PO_2$) 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 O_2 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-O_2 binding characteristics have been thoroughly investigated for only a few elasmobranchs (e.g., *Squalus acanthias* and *Carcharhinus plumbeus*), but a complete picture of O_2 flux in these species has yet to be composed. A recent resurgence in O_2 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-O_2 binding characteristics, CO_2 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 CO_2 excretion in dogfish relies on HCO_3^- 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 HCO_3^- 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 HCO_3^- 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 CO_2 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 HCO_3^- dehydration.

Despite the central roles of O_2 uptake and CO_2 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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