CARBONIC ANHYDRASE IN THE GILLS AND BLOOD OF CHONDRICHTHYAN FISHES

by

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B.Sc. (Hons.), The University of Victoria, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Zoology)

The University of British Columbia
(Vancouver)

January 2018

Abstract

Carbon dioxide (CO_2) is continuously produced as a result of aerobic respiration and must be excreted to maintain internal acid-base balance. Most CO_2 is carried in the blood as HCO_3^- and must be converted back to molecular CO_2 at the respiratory surface to diffuse out into the environment. The uncatalyzed rate of HCO_3^- dehydration is too slow to excrete CO_2 at a physiologically relevant rate and therefore it must be catalyzed by the enzyme carbonic anhydrase. The distribution of carbonic anhydrase in the blood and gills of fish therefore provides important information about general patterns of gas exchange and acid-base balance. Teleost fishes have a fast CA in the red blood cell (RBC), no extracellular CA activity, an endogenous plasma CA inhibitor and a relatively low plasma buffer value so HCO_3^- dehydration is largely restricted to the RBC. Pacific spiny dogfish (*Squalus suckleyi*), however, have a slow RBC CA, extracellular CA activity, no endogenous plasma CA inhibitor, plasma accessible CAIV at the gills and a relatively high plasma buffer value, implying that both the RBC and plasma compartments may contribute to HCO_3^- dehydration. This thesis uses biochemical assays, subcellular localization and immunohistochemistry on blood and gill samples from 13 chondrichthyan species to examine whether the characteristics of the dogfish model of CO_2 excretion apply to chondrichthyan fishes in general. Overall, the results of this study were consistent with the proposed chondrichthyan model of CO_2 excretion because most chondrichthyans had lower RBC CA activity than teleosts, some extracellular CA activity, no endogenous plasma CA inhibitor, higher plasma buffer values and type IV-like CA at the gills. Pacific spiny dogfish had 3x more microsomal CA activity (183 ± 13.2 μmol CO_2 min^{-1} mg $protein^{-1}$) in the gills than the other three species

examined for this trait, indicating that dogfish may not be a representative species to compare with other vertebrate groups. Overall, the results of this thesis suggest that all chondrichthyans have the capacity to use both the plasma and RBC compartments for CO_2 excretion and these data provide important information about general patterns of gas exchange and acid-base balance in fishes.

Lay Summary

Fish represent over half of all vertebrate species and chondrichthyan fishes (sharks, skates, rays and chimaeras) are a particularly diverse fish group that occupy a wide range of habitats and use a variety of strategies to succeed in their environments. Like all vertebrates, chondrichthyans produce carbon dioxide (CO_2) as a byproduct of normal aerobic metabolism. CO_2 must be excreted from the animal and based on research in dogfish, chondrichthyans seem to have a unique strategy for excreting CO_2 because of the distribution of carbonic anhydrase, the key enzyme to this process. This thesis investigates whether the proposed dogfish model of CO_2 excretion applies more broadly to chondrichthyan fishes in general. By using a wide range of techniques, the results of this thesis suggest that all chondrichthyans likely use the same strategy for CO_2 excretion, a finding that provides important information on general patterns of gas exchange in this diverse group.

Preface

Chapter 2 of this thesis is co-authored by Angelina Dichiera, Till Harter, Mike Sackville, Jonathan Wilson, Andrew Esbaugh and Colin Brauner. I conducted all of the research in chapter 2 (research questions, experimental design, experimentation and data analysis) under the supervision of Dr. Colin J. Brauner. Angelina Dichiera and Andrew Esbaugh assisted with sample collection and experimentation during a visit to the University of Texas Marine Science Institute in Port Aransas, TX, USA. Till Harter and Mike Sackville assisted with experimentation for chapter 2. I wrote all chapters of this thesis and received editorial feedback from my committee members, Drs. Colin J. Brauner, William K. Milsom and Chris M. Wood.

All experimental animals were treated according to the University of British Columbia Animal protocol #A11-0235, Vancouver Aquarium Animal Use Protocol #2016-01 and Texas Parks and Wildlife Department Scientific Permit #SPR-0516-121, University of Massachusetts Animal Care Protocol #152.13-06, Pfluger Institute for Environmental Research Ethics Protocol #149-152.12 and California Department of Fish and Wildlife Scientific Collection Permit #SC-2471.

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Acetyl-CoA	acetyl coenzyme A
˚C	degrees Celsius
CA	carbonic anhydrase
CO_3^{2-}	carbonate ion
CO_2	carbon dioxide
GPI-linked	glycosylphosphatidylinositol linkage
H^+	proton
HCO_3^-	bicarbonate ion
H_2CO_3	carbonic acid
K_{cat}	enzyme turnover number
K_m	Michaelis constant
MRCs	mitochondrion-rich cells
MS-222	tricaine methanesulphonate
pK	dissociation constant
PVCs	pavement cells
RBCs	red blood cells
TMAO	trimethylamine oxide
V_{max}	maximal reaction velocity

Acknowledgements

First and foremost, I want to thank my supervisor Dr. Colin Brauner for his unfaltering support and guidance these past three years. It's difficult to articulate how wonderful it was to have a supervisor that gave me both the freedom and confidence to pursue my own ideas and also the helping hand and positive attitude when necessary. I also wish to thank my committee Dr. Chris Wood and Dr. Bill Milsom for their encouragement and helpful comments during all stages of my project.

This thesis would not have been possible without samples provided by Dr. Diego Bernal (University of Massachusetts Amherst), Dr. Chugey Sepulveda (Pflüger Institute for Environmental Research), Phill Morrison, Dr. Chris Wood, Dr. Adalberto Val (National Institute of Amazon Research), Ellen Jung, Dr. Gary Anderson (University of Manitoba), Dr. Greg Goss (University of Alberta), Dr. Martin Haulena and the entire Vancouver Aquarium veterinary team. Special thanks to Dr. Andrew Esbaugh (University of Texas Marine Science Institute) and Dr. Jonathan Wilson (Wilfrid Laurier University) for teaching and hosting me and to Angelina Dichiera for being my fellow shark enthusiast and favourite fishing buddy.

To the Brauner lab, thank you for the great times at lab meetings and Christmas dinners and the support throughout my time here. A very big thank you to the incredible friends I have met during this degree for the endless laughs and words of wisdom. The UBC Zoology community is truly unique and I wish to thank everyone that has been a part of it, both past and present.

Finally, to Ravi Grewal. The unwavering love and support you have shown me mean more than words can describe. You are the best teammate and I can't wait to start our next adventure together.

For Bab

"Off we go, into the wild blue yonder..."

1 Introduction

Chondrichthyan fishes, like all other animals, must excrete metabolically produced CO_2 to maintain internal acid-base balance. The enzyme carbonic anhydrase (CA) is integral to this process and thus the distribution of this enzyme in the blood and at the gills provides important information about the mechanism of CO_2 excretion in different vertebrate groups. Much is known about the teleost model of CO_2 excretion, but knowledge in chondrichthyan fishes is mostly limited to one species. This thesis uses biochemical assays, subcellular localization and immunohistochemical techniques to assess the CA distribution and plasma buffer values of a wide range of chondrichthyan species for a comparison with teleosts. The remainder of this general introduction will introduce chondrichthyan fishes and summarize what is known about CO_2 excretion. It will end with a description of the teleost and dogfish models of CO_2 excretion and then the specific hypotheses and objectives of my thesis.

1.1 Chondrichthyan fishes as a study group

Despite their long and successful evolutionary history, relatively little is known about the physiology, biology and ecology of chondrichthyan fishes when compared to teleost fishes. Fish belonging to the class Chondrichthyes are jawed gnathostomes that have inhabited the world's aquatic environments for around 380 million years (Janvier and Pradel, 2016). Chondrichthyans can be differentiated from their teleost relatives (Class Osteichthyes) by several characteristics including their simple cartilaginous endoskeletons, internal fertilization, paired spiracles and separate internal and external gill openings (Compagno, 1999). Using data from the fossil record, extant chondrichthyan lineages are estimated to be between 66 and 250 million years old, originating during the Triassic, Jurassic or Cretaceous period (Janvier and Pradel, 2016). Modern

chondrichthyans are comprised of 13 extant orders (Figure 1.1) that have evolved remarkably different body forms, reproductive strategies and lifestyles.

Chondrichthyan fishes have invaded almost all aquatic environments on earth, but are more prevalent in some than in others. The highest species richness is found in the tropics, but many species inhabit temperate and some even arctic waters (White and Sommerville, 2010). Many species are demersal, spending most of their life resting on or buried in the sand (e.g. southern stingray, *Dasyatis americana*; Semeniuk and Rothley, 2008). Some species are highly migratory, traveling over 2,000km to find productive foraging habitat (e.g. salmon shark, *Lamna ditropis*; Block et al., 2011), and others undergo diel vertical migrations of hundreds of metres to rest below the thermocline during the day and capture prey at the surface at night (e.g. bigeye thresher shark, *Alopias superciliosus;* Coelho et al., 2015). A few species even inhabit depths of over 1900m (e.g. Portuguese dogfish, *Centroscymnus coelolepis*; Clarke et al., 2001). Despite being found in many different types of environments, chondrichthyan fishes are notoriously underrepresented in freshwater systems.

While it is not fully known why there are so few chondrichthyan species in freshwater systems, the unique osmo- and ionoregulatory strategies between marine and freshwater chondrichthyans may provide some insight. Marine chondrichthyans are osmoconformers with a plasma osmolality of ~1,000mOsm accomplished by retaining urea (~350mM) and trimethylamine oxide (TMAO; ~70mM) as osmolytes in their blood. These species produce small volumes of concentrated urine and use a specialized rectal gland to secrete a highly concentrated NaCl solution (Burger and Hess, 1960). Species that inhabit freshwater for any length of time must abandon this osmoconforming strategy and begin to regulate solute concentrations in the blood. Species that are truly euryhaline, or can live for extended periods of time in both freshwater and marine environments (e.g. Atlantic stingray*, Dasyatis sabina*), decrease plasma

Na^+, Cl^- and urea levels in freshwater, and increase them back to marine levels after gradual transfer to 100% seawater (Piermarini and Evans, 1998). Most stenohaline freshwater chondrichthyans belong to a single family of batoid stingrays found in the Amazon River (Family Potamotrygonidae). These stingrays also maintain their plasma osmolality levels much lower than their marine and euryhaline counterparts, similar to freshwater teleosts. They do not retain urea or TMAO in their blood (Wood et al., 2002), produce large volumes of dilute urine and have a small, non-functional rectal gland (Thorson et al., 1978; Gerst and Thorson, 1977). Freshwater stingrays must take up NaCl from their environment and use their gills to do so, again like freshwater teleosts (Piermarini and Evans, 2001). In fact, these stingrays also use low affinity ion transport systems with low diffusive loss rates to inhabit the ion-poor blackwater of the Rio Negro tributary of the Amazon river (Wood et al., 2002). Only 5% of chondrichthyans can enter or live in freshwater environments and 3-4% live permanently in freshwater, compared to 40% of teleosts (Ballantyne and Fraser, 2013). It is curious that so few chondrichthyans inhabit freshwater systems, but it has been suggested that the decrease in plasma urea with freshwater acclimation may affect proteins that require high levels of urea to function (Ballantyne and Fraser, 2013). This has yet to be investigated, and the limitations to chondrichthyan invasion of freshwater systems is an interesting area for further research. Nevertheless, the osmo- and ionoregulatory mechanisms exhibited by the species that have successfully invaded freshwater habitats are just one example of the many interesting physiological characteristics that allow chondrichthyans to thrive in their environments.

There are several additional physiological characteristics that make chondrichthyans an interesting group to study. For example, Chondrichthyans are ureotelic, meaning they excrete most nitrogenous wastes as urea, and have a specialized salt secreting rectal gland. All chondrichthyans also have a network of jelly-filled electroreceptive pores called Ampullae of

Lorenzini, and several species show physiological adaptations that are unique to the environments that they inhabit. Like tunas and billfishes, certain species of chondrichthyan are regionally endothermic, using the principles of countercurrent heat exchange to maintain the temperature of some tissues above ambient (e.g. shortfin mako shark, *Isurus oxyrhinchus* and porbeagle shark, *Lamna nassus*; Carey and Teal, 1969; Carey et al., 1971). Some chondrichthyans decrease resting oxygen consumption ($M\dot{O}_2$) and the P_{O2} at which they transition from oxyconformers to oxyregulators (P_{crit}) while maintaining hemoglobin-oxygen saturation, allowing them to inhabit shallow coastal waters and grass flats that are chronically or intermittently hypoxic (e.g. epaulette shark, *Hemiscyllium ocellatum*; Carlson and Parsons, 2001; Nilsson and Renshaw, 2004; Speers-Roesch et al., 2012). Chondrichthyans use a suite of physiological characteristics to succeed in their environments, making this group a particularly interesting one in which to study physiological systems. In particular, some of the most important and interesting organs in the respiratory and acid-base physiology of chondrichthyans are the gills, across which gases and ions are exchanged to maintain homeostasis.

1.2 Structure and function of the fish gill

Fish gills are dynamic tissues, simultaneously regulating gas exchange, ions and acid-base balance, and although there are some differences, general gill structure is similar in teleosts and chondrichthyans (Figure 1.2). In both groups, the gills consist of a large surface area and provide a thin barrier that separates the internal and external environments. Each paired gill arch has gill rays that extend laterally from its base and an interbranchial septum made of connective tissue that connect and support the neighbouring filaments (Evans et al., 2005). In chondrichthyan fishes, this interbranchial septum extends from the base of the gill arch to the skin, forming

distinct external gill slits (Wegner, 2016). Conversely, in teleost fishes the interbranchial septum is reduced, only extending to the base of the filaments. This modification allows teleost gills to be much more flexible than those of chondrichthyans. Four pairs of holobranchs (cranial and caudal hemibranchs extending from the same arch) are present in both chondrichthyan and teleost fishes, but in chondrichthyans there is also an additional pair of caudal hemibranchs on the first gill arch (Evans et al., 2005; Wegner, 2016; Evans et al., 2005). Extending outwards from the base of the gill arch are the filaments, each supplied with blood from the filamental artery, and lamellae, structures perpendicular to the long axis of filaments that increase surface area (Evans et al. 2005). Water flows between lamellae and countercurrent to blood flow to maximize gill oxygen loading. Distinct pillar cells connect the two sides of the respiratory epithelium and help to guide blood flow through the lamellae. The gills of both teleost and chondrichthyan fishes are highly efficient structures that play many roles in maintaining homeostasis and are composed of many different cell types to accomplish these tasks.

In general, both chondrichthyan and teleost gills are composed mainly of five types of cells: pillar cells, pavement cells (PVCs), mitochondrion-rich cells (MRCs), goblet (mucous) cells and neuroepithelial cells (Wilson and Laurent, 2002). PVCs are the most abundant cell type in both species, covering over 90% of gill surface area both on the filaments and lamellae. The lamellar epithelium is thinner (1-3 cell layers thick) than the filamental epithelium (3 or more cell layers thick), and is lined with PVCs which constitute the primary surface for gas exchange (Wilson and Laurent, 2002). MRCs are large and rarely found as part of the lamellar epithelium, instead comprising a large proportion of interlamellar filament epithelial cells. These cells are superficially similar between teleosts and chondrichthyans, both having high mitochondrial densities, a basal nucleus and a dense tubulovesicular system (Wegner, 2016). MRCs in marine teleosts are thought to function in Na^+ and Cl^- efflux (Wilson et al., 1997; Piermarini and Evans,

2001; Evans et al., 2004, Tresguerres et al., 2006) and are surrounded by accessory cells and leaky junctions for paracellular Na^+ transport (Laurent, 1984; Wilson and Laurent, 2002; Evans et al., 2005). Conversely, chondrichthyan and freshwater teleost MRCs are usually found between two pavement cells with tight junctions and are thought to function mostly in acid base balance and bidirectional Na^+ and Cl^- transport (Wilson et al., 1997, 2002; Wright and Wood, 2016). Goblet cells contain secretory granules and are found at the afferent and efferent edges of filaments and in the interlamellar space both in teleosts and chondrichthyans, but rarely on lamellae themselves due to their large size (Wegner, 2016). Neuroepithelial cells are found deep in the filament epithelium of both teleosts and chondrichthyans and are thought to function in oxygen sensing and regulating blood flow (Laurent, 1984). Although there are slightly different distributions and functions between teleost and elasmobranch fishes, the five cell types of the gill play important roles in gas exchange, acid base balance and ion regulation. In particular, the gills are vital to oxygen uptake and CO_2 excretion, processes that result from the use of aerobic respiration.

1.3 Carbon dioxide excretion

Aerobic metabolism is a pre-requisite for vertebrate life and its main goal is to match energy supply with energy demand by producing enough adenosine triphosphate (ATP) to fuel metabolic processes. Oxygen is taken up across the gills and bound to hemoglobin in red blood cells (RBCs) after which it is transported to the tissues. This oxygen is then used in oxidative phosphorylation where an electrochemical proton gradient is used to phosphorylate ADP to ATP (McClelland, 2011). During the process of aerobic metabolism, carbon dioxide (CO_2) is produced

through the conversion of pyruvate to Acetyl-CoA and during the Krebs cycle (McClelland, 2011). CO_2 then diffuses out of the tissues and into the bloodstream.

Since CO_2 is constantly produced and acts as a weak acid in solution, it must be constantly excreted to maintain internal acid-base balance. CO_2 hydrates (combines with water) and bicarbonate dehydrates (produces water) in water according to the following reaction:

Equation 1: $$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

The formation of carbonate in the second dissociation step is not significant at physiological pH because of its high dissociation constant (pK_2 = 10.5 at 10˚C; Perry 1986), so at physiological pH, the reaction is often simplified to:

Equation 2: $$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

It is known that CO_2 is formed in the tissues and must be transported to the respiratory surface where it is excreted, but the details of this process can vary among vertebrate groups. Nevertheless, all animals that utilize aerobic metabolism must excrete CO_2 and research on the pathways involved in CO_2 excretion have provided valuable information on gas exchange and acid-base balance in fishes.

CO_2 follows a series of diffusive and convective transport steps before it is excreted across the gills, the first step being the diffusion of CO_2 out of the tissues and into the RBC where CO_2 hydration occurs (see Figure 1.3 for a visual representation). Within the RBCs, the Bohr and Haldane effects play major roles in regulating gas transport and exchange and it is well known that the magnitude of these effects vary among vertebrate groups. High CO_2 concentrations in the blood at the tissues decrease hemoglobin's affinity for oxygen, elevating P_{O2} and thus the driving

force for oxygen diffusion into the tissues (Bohr effect; Bohr et al., 1904). As oxygen is unloaded to the tissues, the affinity of hemoglobin for protons and CO_2 is increased and consequently, deoxygenated blood has a higher CO_2 content (Haldane effect; Christiansen et al., 1914), thereby facilitating CO_2 removal from the tissues. Thus, at the tissues, there is an interaction between O_2 and CO_2 transport where one process facilitates the other. After CO_2 is hydrated within the RBC to form HCO_3^- and H^+, HCO_3^- is transported into the plasma by the Cl^-/HCO_3^- exchanger where it remains until the blood reaches the gills. It is important to note that while CO_2 is predominantly transported as HCO_3^- (90-95%; Eddy, 1974, Wood et al., 1977, Heming, 1984), some is also transported as carbamino CO_2 (5%, Heming, 1984) and carbonate (CO_3^{2-}) ions (0.03% Heming, 1984). A small percentage is even transported as molecular CO_2 (4%, Perry, 1986). However, given that the majority is HCO_3^-, other components will not be described in detail. Overall, CO_2 excretion in fishes is a dynamic process, and in particular the dehydration of HCO_3^- to molecular CO_2 at the gills is a critical step that allows CO_2 to be excreted at a physiologically relevant rate.

Upon arrival at the gills, HCO_3^- is converted back to molecular CO_2 for diffusion out of the animal. In order for this to occur, two conditions must be met: HCO_3^- dehydration must occur faster than blood moves through the gills and there must be a source of protons to combine with HCO_3^-. Under uncatalyzed conditions, the rate constant of HCO_3^- dehydration is between 25 and 90 seconds at physiological pH and temperature (Edsall, 1969; Heming, 1984), yet blood transit time through the gills is only 0.5-6.5 seconds (Edsall, 1968; Cameron and Polhemus, 1974; Randall and Daxboeck, 1984; Bhargava et al., 1992). Therefore, the uncatalyzed rate is insufficient to excrete CO_2 at an appreciable rate and the enzyme carbonic anhydrase (CA) must be used to increase the reaction rate up to 25,000 times (Gilmour et al., 2011). However, the concentration of CA itself is not thought to be limiting and the rate-limiting step of CO_2 excretion is likely the rate of Cl^-/HCO_3^- exchange bringing HCO_3^- into the red blood cell (Perry 1986). The

protons needed for HCO_3^- dehydration are provided by the buffers of the respective compartment. If only the RBC is used, then the protons come from hemoglobin, where protons are either released during oxygenation (Haldane effect) or are provided by other sources (non-oxygenation dependent). Protons can also be supplied from organic phosphates within the RBC. If the plasma compartment is used, there must be sufficient plasma buffering capacity to provide protons for HCO_3^- dehydration and most of this is provided by plasma proteins. Once produced, molecular CO_2 diffuses down its partial pressure gradient into the environment (≥85% of total excretion; Perry, 1986) and a small amount (≤15%; Perry, 1986) is hydrated within epithelial cells to form HCO_3^- and H^+ which are exchanged across the apical membrane for Cl^- and Na^+, respectively. The distribution of CA in the gills and blood of fishes along with the buffer values of the blood compartments will provide insight on how these animals excrete CO_2.

1.4 CA and its role in CO_2 excretion

CA is one of the fastest and most widely distributed enzymes known. Based on its varied uses in bone resorption, calcification, photosynthesis, cell growth, ion transport, acid base transport, metabolic processes and respiratory gas transport, it is also thought to be among the earliest enzymes to have evolved (Tashian, 1989; Chegwidden and Carter, 2000; Esbaugh and Tufts, 2006). First discovered in mammalian erythrocytes (Meldrum and Roughton, 1933), this enzyme increases the reaction rate up to 25,000-fold (Gilmour, 2011) catalyzing the reversible reaction:

Equation 3: $$CO_2 + OH^- \leftrightarrow HCO_3^-$$

CA eliminates the slower formation of H_2CO_3 in the CO_2 hydration and dehydration reactions. It uses a zinc-bound hydroxide ion to dehydrate bicarbonate and hydrate CO_2 quickly according to the following equations:

Equation 4: $CA\text{-}Zn + H_2O \leftrightarrow CA\text{-}ZnOH^- + BH^+$

Equation 5: $CA\text{-}ZnOH^- + CO_2 \leftrightarrow CA\text{-}Zn + HCO_3^-$

Specifically, a water molecule is split at the active site to create a zinc-bound hydroxide ion and then a mobile non-bicarbonate buffer complex (BH^-) removes the resultant proton. A CO_2 molecule is then brought into the active site pocket of the CA where it undergoes a nucleophilic attack by the zinc-bound hydroxide ion, forming a bicarbonate ion (Silverman and Lindskog, 1998; Christianson and Fierke, 1996). A water molecule then replaces the bicarbonate in the active site, and the zinc-bound hydroxide ion is regenerated by the removal of a proton via a proton shuttling mechanism (Tu and Silverman, 1989). This proton removal is the rate-limiting step of the mechanism and is highly dependent on the availability of buffer agents in the cytoplasm (Lindskog and Silverman, 2000).

There are at least three (Hewett-Emmett and Tashian, 1996; Hewett-Emmett, 2000) and potentially as many as five (Xu et al., 2008) genetically unrelated classes of CA isoforms (α, β, γ, δ, ξ), of which the α-CA family of vertebrate isoforms is the most well-studied. The mammalian α-CA gene family consists of three groups of CA isoforms with different sequences, biochemical properties and subcellular location. The cytoplasmic forms (CA I, II, III, V, VII and XIII) are found in the cytoplasm of various tissues, except for CA V, which is confined to mitochondria. The fastest of the cytoplasmic forms is CA II, which has a turnover number (the number of times an enzyme converts substrate to product per unit time; K_{cat}) of 1×10^6 s^{-1} and is generally found in RBCs (Swenson 2000). Cytoplasmic CA isozymes from mammalian and non-mammalian

vertebrates do not group together when phylogenetic analyses are performed, indicating that the high activity isozymes found in fish differ from those in mammals (Lund et al. 2002; Esbaugh et al. 2004a; Esbaugh et al. 2005). Key elements of the catalytic mechanisms, however, only show 2-4 different amino acid between mammalian CA II and representative cytoplasmic isozymes from other vertebrate groups including agnathans, teleosts, amphibians and birds, suggesting that the catalytic mechanism is conserved among vertebrate groups (Esbaugh et al., 2006). The membrane-bound forms (CAIV, IX, XII, XIV and XV) are associated with plasma membranes of many different tissues (Sly, 2000). These membrane-bound forms include CAIV, one of the two isozymes anchored to membranes via a glycosylphosphatidylinositol (GPI) linkage and involved in HCO_3^- dehydration at the respiratory surface. In contrast to cytoplasmic isozymes, membrane-bound CA isozymes from non-mammalian vertebrates are closely grouped with their mammalian isozymes, suggesting that it is CAIV that is observed in non-mammalian vertebrate groups (Esbaugh et al., 2006). Lastly, the CA-related proteins (CA VIII, X, and XI) have lost the hydration and dehydration functions of other isozymes, and their function is unknown (Tashian et al. 2000). Given that there are phylogenetic differences between vertebrate groups and many isozymes with different biochemical functions and subcellular localizations, examining the distribution and activity of CA provides insight into which CA isoforms are present in chondrichthyans.

1.5 CA distribution in fishes

The description of CA distributions in respiratory tissues has been modified many times over the course of its investigation. Since the location of CA determines the site where HCO_3^- can be converted to CO_2 for excretion, examining distribution patterns in gills and blood is important

for understanding general patterns of gas transport and the evolution of such processes. After the discovery of CAI and II in mammalian RBCs (Meldrum and Roughton, 1933), it was thought that all CA was absent from the plasma of mammals and lower vertebrates (Maren, 1967). This view changed upon the discovery of CAIV in the endothelial membrane of mammal (Crandall and O'Brasky, 1978; Effros et al., 1978; Klocke 1980; Whitney and Briggle, 1982) and turtle (Stabenau et al., 1996) lungs that is available to catalyze HCO_3^- dehydration in the plasma. Further investigations in fish, however, revealed the absence of such plasma-accessible CA at the gills despite similarly high levels of RBC CA (see review by Gilmour and Perry, 2010). As a result, the idea that different vertebrate groups have different distributions of CA at the respiratory surface emerged and has since been a highly investigated topic in comparative physiology.

1.6 The teleost model of CO_2 excretion

Fishes represent over half of all vertebrates (Nelson, 1984), and teleost fishes in particular are the most well-studied in terms of CO_2 excretion. Specifically, CO_2 excretion has been most comprehensively researched in rainbow trout (*Oncorhynchus mykiss*). The teleost model of CO_2 excretion is characterized by a lack of membrane-bound plasma-accessible CA activity at the gills, the reason for which is still debated (Figure 1.3; Table 1.1; Henry et al., 1997; Gilmour et al., 2001, 2002, Stabenau and Heming, 2003; Georgalis et al., 2006). Since most teleost hemoglobins have strong Haldane and Bohr effects and a low buffering capacity, O_2 transport and CO_2 excretion are tightly linked through the production and consumption of protons in the RBC (Jensen 1986; Brauner and Randall, 1998). Teleosts also have a pH sensitive hemoglobin (Root effect) where changes in pH can negatively affect O_2 uptake (Root, 1931). To protect

against this, many teleosts have a ß-adrenergic sodium proton exchanger (ßNHE) on the red blood cell membrane that is stimulated by catecholamines (Rummer and Brauner, 2011). Under stress, catecholamines activate the ßNHE which pumps protons out of the RBC, thereby protecting intracellular pH and O_2 uptake at the gills in the presence of a blood acidosis (Randall et al., 2014). The presence of CA in the plasma would short-circuit this response and compromise oxygen loading at the gills by rapidly converting excreted protons back into CO_2 that would diffuse back into the RBC (Rummer and Brauner, 2011; Rummer et al., 2013). Thus, one theory for the lack of plasma-accessible membrane-bound CA at the gills is to protect O_2 uptake. There are also other theories, such plasma HCO_3^- dehydration reducing HCO_3^- cycling through the RBC, thereby limiting the HCO_3^- available to combine with protons and causing an acidosis (Lessard et al., 1995). While the exact reason for the lack of membrane-bound plasma-accessible CA is unknown, its absence is likely important for maintaining oxygen transport and/or acid-base balance in the presence of a pH-sensitive hemoglobin.

Not surprisingly, teleosts also lack soluble CA in the plasma (Gilmour et al., 1997; 2001) and have an endogenous plasma CA inhibitor (Henry et al., 1997). In mammals, this inhibitor is thought to inhibit the activity of CA arising from normal cell lysis because mammals have fragile, anucleate RBCs (Booth, 1938; Van Goor, 1948; Hill, 1980; Roush and Fierke, 1992). Fish have more robust, nucleated RBCs, so this inhibitor could have many functions in this group, such as protecting intracellular pH and regulating cell volume (Lessard et al., 1995). The function of the CA inhibitor could also be independent of gas exchange, and it has been hypothesized to function in zinc transport and recycling. Porcine plasma CA inhibitors have 62% primary sequence homology to transferrin, an essential component of iron transport and metabolism (Henry and Heming, 1998). In this regard, the inhibitory functions of the plasma CA inhibitor would be incidental to their primary function of transporting and metabolizing zinc

(Henry et al., 1997; Henry and Heming, 1998). Regardless of its origin or function, when coupled with the lack of membrane-bound plasma-accessible CA at the gills, this inhibitor makes the plasma compartment an unfavourable environment for CO_2 production at the gills in teleosts, leaving the RBC as the only site of CO_2 production.

Teleost fishes generally have a high concentration of a fast turnover CA isoform in the RBC ([CA] = 1.1mmol L^{-1}, k_{cat} = 30.3-70 e^4s^{-1} for rainbow trout; Maren et al., 1980; Gervais and Tufts, 1999; Esbaugh et al., 2005) resulting in a high overall CA activity (155-513 mmol CO_2 min^{-1} ml RBC^{-1}; Henry et al., 1997; Esbaugh et al., 2005). Species can differ in their absolute RBC CA activity, but these differences are thought to result from differences in concentration, not in catalytic activity (Esbaugh et al., 2004). Since CA is restricted to the RBC, each HCO_3^- must enter via the Cl^-/HCO_3^- exchanger and this is the rate-limiting step of CO_2 excretion in teleosts (Perry and Gilmour, 1993; Desforges et al., 2001). The teleost model of CO_2 excretion was originally thought to apply to all fishes, but further research discovered a notable exception.

1.7 The dogfish model of CO_2 excretion

In contrast to other fish groups that have been examined, evidence in the spiny dogfish (*Squalus suckleyi*, previously *Squalus acanthias)* suggests that chondrichthyans use both the RBC and plasma compartments for CO_2 excretion (Figure 1.3). Over 20 years of investigation, a picture has emerged of CO_2 excretion in dogfish that is unlike that which has been observed in any other group of aquatic vertebrates (see Table 1.1 for a comparison with teleosts). Characterized by plasma-accessible membrane-bound CA at the gill, plasma CA activity, no endogenous plasma CA inhibitor and low RBC CA activity, this model of CO_2 excretion has

received much attention in the literature but it remains to be seen whether it is representative of all chondrichthyan fishes.

S. suckleyi has membrane-bound plasma accessible CA activity at the gills that has been identified as the GPI-linked isoform CAIV (Henry et al., 1997; Gilmour et al., 1997, Gilmour et al., 2001; Wilson et al., 2000). Pharmacological inhibition of CAIV using a perfused head preparation showed an acid-base disequilibrium in post branchial perfusate, suggesting that this pool of CA does contribute to CO_2 excretion (Wilson et al., 2000). Subsequent *in vivo* experiments confirmed this finding and after treatment with a low dose of the extracellular CA inhibitor benzolamide, a significant reduction in arterial-venous total CO_2 concentration was observed, as well as a significant increase in PCO_2 and a blood acidosis (Gilmour et al., 2001). It is now widely accepted that CAIV contributes to CO_2 excretion in dogfish and there have been studies on several blood characteristics to support this unique model of CO_2 excretion.

S. suckleyi lacks an endogenous CA inhibitor in the plasma (Henry et al., 1997) and there is evidence for circulating CA activity in separated plasma from dogfish (*Scyliorhinus canicula*; Wood et al., 1994; Perry et al., 1996). HCO_3^- dehydration that occurs in the plasma can bypass the rate-limiting Cl^-/HCO_3^- exchange or act as a reserve capacity for CO_2 excretion during exercise. While this pool of CA was originally thought to account for up to 50% of CO_2 excretion (Wood et al. 1994), the estimate of plasma CA contributions to total CA activity was calculated by Henry et al. (1997) as only ~0.1%. Circulating CA activity in the plasma is now thought to come from normal RBC lysis and is detectable in the plasma due to the absence of an endogenous CA inhibitor. Nevertheless, there is some CA activity in separated plasma that has the potential to supplement CO_2 excretion from the RBC.

Like all other vertebrates examined, CA is found in the RBCs of *S. suckleyi*, but its activity is lower than in teleosts (30.8 mmol CO_2 min^{-1} ml $RBCs^{-1}$; Henry et al., 1997). In *S.*

suckleyi, CA concentrations ([CA] = 0.024 mmol L^{-1}; Maren et al., 1980; Swenson and Maren, 1987) and k_{cat} values (k_{cat} = 2.5 e^4 s^{-1}; Maren et al., 1980) are much lower than those of rainbow trout ([CA] = 1.1mmol L^{-1}, k_{cat} = 30.3-70 e^4s^{-1} for rainbow trout; Maren et al., 1980; Gervais and Tufts, 1999; Esbaugh et al., 2005). By cloning CA from dogfish blood and examining the active site, Gilmour et al. (2007) determined the substitution of a serine for a histidine residue in the proton shuttling ligand. This histidine residue is part of the rate-limiting step of CA by regenerating the zinc-bound hydroxide ion and replacing this amino acid with one that cannot transfer protons decreases enzyme activity (Tu and Silverman, 1989; Stams and Christianson, 2000; Lindskog and Silverman, 2000). In addition, CO_2 excretion in dogfish gills is highly efficient compared to teleosts (dogfish: $PaCO_2$ ~1 Torr, $CaCO_2$ ~4mmol L^{-1}; teleosts: $PaCO_2$ ~3 Torr, $CaCO_2$ ~6-8mmol L^{-1}; Wood et al., 1982, 1994), yet they have a very small or absent Haldane effect (Wood et al., 1994). HCO_3^- dehydration in the RBC therefore does not benefit from the release of oxylabile protons during hemoglobin oxygenation. This fact, coupled with low RBC CA activity, may decrease the reliance on the RBC for CO_2 excretion. Therefore, the efficient CO_2 excretion seen in dogfish is likely a result of plasma HCO_3^- dehydration and this process relies on the buffer value of the plasma.

Not only is a CA source necessary for HCO_3^- dehydration in the plasma, a proton source is also needed to combine with HCO_3^-. Non-bicarbonate plasma buffer values are the major non-CA characteristic that is important in determining whether a species can use the plasma compartment for CO_2 excretion. In mammals, a low relative plasma buffer value (~15-20% of whole blood value; Heisler, 1986) may represent the basis for pulmonary CAIV being involved in <10% of total CO_2 excretion (Bidani and Heming, 1991; Heming and Bidani, 1992). Teleost plasma accounts for ~20-40% of total blood buffer values but the absence of CAIV at the gill prohibits plasma HCO_3^- dehydration (Table 1.1; Gilmour et al., 2002). In dogfish and skate,

however, plasma accounts for anywhere from 40-70% of whole blood buffering (Table 1.1; Lenfant and Johansen, 1966; Graham et al., 1990; Tufts and Perry, 1998; Gilmour et al., 2002). When coupled with CA activity, the buffer value of the plasma allows it to contribute to CO_2 excretion (Figure 1.3).

Many chondrichthyan species are large and difficult to sample as well as threatened or endangered, making research challenging. Furthermore, physiological studies often use samples of blood or tissues that require close contact with individuals in a research facility or in the wild. As a result, most of the research on CO_2 excretion is restricted to *S. suckleyi* and it is unclear whether the dogfish model of CO_2 excretion persists throughout the chondrichthyan group. In addition, chondrichthyan fishes have a variety of body forms, live in diverse environments, display many unique physiological features and span approximately 400 million years of evolution so it is important to understand their basic patterns of gas exchange and acid-base balance.

1.8 Thesis objectives

The general objective of this thesis was to investigate whether a wide range of chondrichthyan fishes have the capacity to utilize the same model of CO_2 excretion as the Pacific spiny dogfish (*Squalus suckleyi*). This thesis incorporates biochemical assays, subcellular localization and immunohistochemical techniques to assess CA and accessory characteristics in the RBCs, plasma and gills according to the following three objectives:

Objective 1: To assess the CA activity in the RBCs and plasma of chondrichthyan species.

I hypothesize that if all chondrichthyans utilize the plasma for CO_2 excretion, they will have CA characteristics in the blood that are more similar to dogfish than to rainbow trout.

Provided that the plasma and RBC both contribute to CO_2 excretion in dogfish, decreased selective pressure for a high activity enzyme may have allowed the RBC to express a lower activity CA. I predicted that if all chondrichthyans use the same CO_2 excretion strategy as dogfish, they too would have a lower CA activity in the RBC because the plasma would supply some of the necessary CA activity.

Rainbow trout have an endogenous CA inhibitor that prevents the plasma from contributing to CO_2 excretion. I predicted that other chondrichthyans, like the dogfish, lack this endogenous plasma inhibitor because it would be detrimental to using the plasma compartment for CO_2 excretion.

There is some circulating activity of CA in dogfish plasma that is thought to contribute (if minimally) to CO_2 excretion. I predicted that if other chondrichthyans do not have an endogenous plasma inhibitor, they will have some CA activity present in the plasma.

Objective 2: To compare the non-bicarbonate plasma buffer values of chondrichthyan species to teleosts.

Sufficient protons are necessary to combine with HCO_3^- and form molecular CO_2 at the gills. Dogfish plasma has a higher non-bicarbonate buffer value than teleosts, therefore I predicted that other chondrichthyan fishes would show a higher plasma buffer value than rainbow trout to provide protons for HCO_3^- dehydration.

Objective 3: To investigate the presence of plasma-accessible, membrane-bound CA in the gills of chondrichthyan fishes.

Dogfish have GPI-linked, plasma accessible CAIV at the gills that allows the plasma to contribute significantly to CO_2 excretion. Teleost fishes do not have this trait and therefore I hypothesize that if other chondrichthyans have a similar capacity to utilize the plasma for CO_2 excretion, they will also have GPI-linked plasma accessible CAIV at the gills.

2 Carbonic anhydrase in the gills and blood of chondrichthyan fishes

2.1 Introduction

Carbon dioxide (CO_2) is a byproduct of aerobic metabolism that must be excreted. Since it is a weak acid, 90-95% of CO_2 is transported in the blood as bicarbonate ions (HCO_3^-) at normal blood pH (Perry et al., 1986) then dehydrated back to molecular CO_2 at the gills of fish. Blood transit time through the gills is short (0.5-6.5 seconds; Edsall, 1968; Cameron and Polhemus, 1974; Randall and Daxboeck, 1984; Bhargava et al., 1992) yet the uncatalyzed dehydration rate constant of HCO_3^- is 25-90 seconds at physiological pH and temperature (Edsall, 1969; Heming, 1984). CO_2 excretion is thus dependent on the catalyzed conversion of HCO_3^- to CO_2 by the enzyme carbonic anhydrase (CA). CA is typically at high concentrations in the red blood cell (RBC) where there is an interaction between O_2 and CO_2, but some groups have CA at other locations in the gills and blood that have the potential to contribute to CO_2 excretion.

Carbonic anhydrase is present in the pulmonary capillaries of mammals (Whitney and Briggle, 1982; Waheed et al., 1992), birds, reptiles and amphibians (Stabenau et al., 1996; Stabenau and Heming, 1999; Stabenau and Vietti, 2002; Stabenau and Heming, 2003) and is available to catalyze plasma CO_2 reactions (Zhu and Sly, 1990). It has been identified as CA IV, a high activity isoform that is bound to membranes by a glycosylphosphatidylinositol (GPI) anchor. However, pulmonary CA IV in mammals only accounts for <10% of total CO_2 excretion due to the low relative non-bicarbonate buffer value of plasma and therefore limited availability of protons for HCO_3^- dehydration. Additionally, some mammals have an endogenous plasma CA inhibitor that further prevents plasma from contributing to CO_2 excretion (Rispens et al., 1985; Hill 1986; Roush and Fierke, 1992). Therefore, all HCO_3^- is dehydrated in the RBC and this

pattern of CO_2 excretion was thought to be typical of all vertebrates, but it is now known to differ in fish.

Teleost fishes lack plasma-accessible CA at the gills (Henry et al., 1988, 1993, 1997; Sender et al., 1999; Gilmour et al., 2001, 2002, Stabenau and Heming, 2003; Georgalis et al., 2006), yet similar to mammals they have an endogenous plasma inhibitor and utilize only the RBC for CO_2 excretion. It is important to note that CO_2 excretion in teleosts is tightly coupled to O_2 transport through the RBC. Teleosts have a pH-sensitive hemoglobin (strong Root and Bohr effects) and a low hemoglobin buffer value, where changes in pH can negatively affect O_2 loading at the gills (Bohr et al., 1904; Root, 1931; Jensen and Weber, 1985; Brauner and Randall, 1996). When CO_2 is hydrated at the tissues, the resultant proton facilitates oxygen unloading from hemoglobin. Conversely, the proton released upon hemoglobin oxygenation is consumed by HCO_3^- dehydration for CO_2 excretion (Brauner and Randall, 1996). Teleosts also have a protective mechanism for RBC pH regulation during stress, the ß-adrenergic sodium proton exchanger (ßNHE) that actively pumps protons out of the RBC in exchange for Na^+ when activated by catecholamines (Mahé et al., 1985). Plasma accessible CA is absent in the gills of teleosts and would otherwise short-circuit RBC ßNHE and compromise O_2 loading at the gills (Rummer and Brauner, 2011; Rummer et al., 2013; Randall et al., 2014). Thus, teleosts have a fast RBC CA with a high turnover number that is sufficient for CO_2 excretion (Maren et al., 1980; Henry et al., 1997; Esbaugh et al., 2005). This teleost model of CO_2 excretion was thought to apply to all fishes, but investigation into the spiny dogfish (*Squalus acanthias = suckleyi*), yields a pattern that is somewhat intermediate between mammals and teleosts.

The "dogfish model of CO_2 excretion" is characterized by two pools of CA activity that are accessible to plasma HCO_3^- reactions. Like mammals, dogfish possess membrane-bound CA at the gill that is susceptible to treatment with phosphatidylinositol-phospholipase C (PI-PLC), an

enzyme that cleaves the GPI anchors characteristic of CAIV (Henry et al., 1997; Gilmour et al., 2001) and unaffected by sodium dodecyl sulphate (SDS), a detergent to which the disulphide bonds of CAIV are resistant (Whitney and Briggle, 1982; Waheed et al., 1996). *In vivo* experiments have confirmed that plasma-accessible CA in dogfish gills contributes significantly to CO_2 excretion (Gilmour et al., 1997; Wilson et al., 2000; Gilmour et al., 2001; Gilmour and Perry, 2004). Buffer values of dogfish plasma account for 40-70% of whole blood buffer value (Gilmour et al., 2002), whereas mammal plasma accounts for only 15-20% (Heisler, 1986), indicating that protons are not as limiting to CO_2 excretion in dogfish plasma. Contrary to both mammals and teleosts, dogfish lack an endogenous plasma CA inhibitor (Henry et al., 1997). This allows some circulating CA to exist in the plasma that is likely the result of RBC lysis during normal turnover (Henry et al., 1997). Furthermore, dogfish have a lower concentration of RBC CA with a lower turnover number (k_{cat}) than rainbow trout (Maren et al., 1980; Swenson and Maren, 1987). Further research into the amino acid sequence of dogfish RBC CA revealed that a serine residue is substituted for his-64 in the proton shuttling mechanism, similar to the substitution seen in mammal RBC CA (Gilmour et al., 2007). Together, these findings suggest that dogfish use a different strategy than both mammals and teleosts for CO_2 excretion by using both the plasma and RBC for HCO_3^- dehydration at the gills.

It is assumed that all chondrichthyans utilize the same strategy for CO_2 excretion as dogfish, but only a small number of species have been tested. This fact is not surprising because most chondrichthyan fishes are difficult to keep in captivity and samples from wild individuals are rare. However, chondrichthyan fishes span 400 million years of evolution, show impressive diversity in morphology and behaviour and use a variety of physiological strategies to inhabit almost every type of aquatic environment so it is possible that there could be great variation in the strategy used for CO_2 excretion. To test this, I used biochemical, subcellular localization and

immunohistochemical techniques to assess the activity and distribution of CA in blood and gill tissue from a wide range of chondrichthyan species. Specifically, I quantified RBC and plasma CA activity, the presence and potency of a plasma CA inhibitor, plasma buffer values, and the presence or absence of plasma accessible CA through measurement of membrane-bound CAIV at the gills and immunohistochemistry. These analyses were conducted on blood from 13 chondrichthyan species and gills from 4 of them with two teleost comparisons. This study found that in general, chondrichthyans show similar CO_2 excretion characteristics to dogfish, which differ from teleosts, but that there is more variation within the group than expected. Investigations such as this are important to advance our understanding of general patterns of CO_2 excretion among vertebrate groups. Since chondrichthyans are a basal vertebrate group, this study will also contribute to our growing knowledge of the evolution of gas exchange and acid-base balance in vertebrates.

2.1 Methods

2.1.1 Sample collection and processing

Samples were collected from thirteen chondrichthyan species and two teleost species (Table 2.1). Care was taken in the selection of species to ensure the diversity of chondrichthyan fishes was captured. All characteristics were also measured in Pacific spiny dogfish as an internal control and rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) teleost comparisons.

Animals were anaesthetized via immersion in tricaine methanesulfonate (MS-222; 0.1g/L for anaesthesia, 0.6g/L for euthanasia). Blood was withdrawn via caudal puncture (and dorsal venous network in stingrays) and centrifuged at 10,000 g for 3 minutes to separate plasma and red blood cells. Plasma samples were immediately frozen at -80˚C whereas red blood cells were

resuspended in isotonic saline (1,000mOsm/L NaCl for most chondrichthyans, 300mOsm/L NaCl for teleosts and *Potamotrygon spp.*), centrifuged at 10,000 g for 3 minutes and the supernatant was discarded. Samples were washed in this manner three times before freezing. Following the collection of blood samples, species for which gills were collected were perfused with heparinized (45 IU/ml) isotonic saline to remove all red blood cells. A catheter made of P50 tubing with a flared end was inserted and sutured into the conus arteriosus (bulbus arteriosus for teleosts) and at least 300mL of isotonic saline was slowly pushed through. One perfused gill arch from each animal was fixed according to one of three fixation protocols (Table 2.2). All remaining arches were dissected out of the animal, frozen in liquid nitrogen and stored at -80˚C until use.

2.1.2 Electrometric ΔpH assay

Carbonic anhydrase activity was measured using the electrometric ΔpH assay as described by Henry (1991). A reaction vessel containing 6mL of ΔpH assay buffer (in mmol L^{-1}: 225 mannitol, 75 sucrose, 10 TRIS base adjusted to pH 7.40 with 10% H_3PO_4) was chilled to 4˚C and kept well mixed by a magnetic stir bar and associated motor. A sample of enzyme source (RBC, plasma, gill fraction) was added, the reaction was initiated by 100µL CO_2 saturated water, and the change in pH observed over time using a GK2401C electrode and PHM 84 pH meter (both Radiometer, London, ON, Canada) connected to a BioPac Data acquisition system and Acknowledge® data acquisition software version 3.7.3. The rate of CO_2 hydration in absence of any CA source (uncatalyzed rate) was subtracted from the observed rate and the buffer capacity was taken into account to convert the observed rate from pH units min^{-1} to mol H^+ min^{-1}. According to the stoichiometry of Equation 2 (see Chapter 1), mol H^+ min^{-1} is equal to mol CO_2

min^{-1} and the rates were expressed per millilitre of CA source based on the volume added to the reaction vessel.

2.1.3 Carbonic anhydrase characteristics in chondrichthyan blood

To assess red blood cell CA activity, frozen RBCs were thawed on ice and diluted 100x in ice-cold distilled water to ensure cell lysis. The ΔpH assay was used to measure the rate of CO_2 hydration in the presence of RBC lysate. The rate of CO_2 hydration of Pacific spiny dogfish lysate was also assessed with increasing amounts of CO_2-saturated water to examine whether 100μL was enough to approach the maximal rate. Protein content was measured using the Bio-Rad standard (Bradford) protein assay with bovine serum albumin (Bio-Rad, Mississauga, ON, Canada) as a standard and RBC CA activity has been expressed per mg protein. To assess plasma CA activity, frozen plasma samples were thawed on ice and 100μL was added to the ΔpH assay. Hemoglobin content was measured using the standard cyanmethemoglobin method (Sigma-Aldrich, Oakville, ON, Canada) as a proxy for haemolysis contamination in the samples. Total plasma protein was also measured using the same method as above. Presence or absence of an endogenous CA inhibitor was assessed by adding 100μL separated plasma to the reaction vessel that already contained a sample of red blood cell lysate. The percent inhibition of RBC lysate activity by 100μL plasma was measured. Since urea and trimethylamine oxide (TMAO) are major osmotic constituents of chondrichthyan blood, their effect on the CA activity of *S. suckleyi* plasma and RBC lysate was tested by adding 340mmol L^{-1} urea and 70mmol L^{-1} TMAO to the reaction vessel (Withers et al. 1994).

2.1.4 Plasma buffer values

Given that the blood samples were coming from various locations and were mostly previously frozen, traditional tonometry methods for assessing plasma buffer values were not possible. Instead, plasma titrations were conducted on diluted separated plasma (200µl in 4.5ml ddH_2O). A TIM865 Titration Manager (Radiometer, London, ON, Canada) connected to Titramaster 85 Data Collector software version 5.2.0 was used to acidify the sample to pH 4 using 0.1N HCl (Fisher Scientific, Burlington, ON, Canada) then alkalinize from pH 4 to 8 using 0.1N NaOH (Fisher Scientific, Burlington, ON, Canada). In the subsequent titration curves, the amount of acid added for each observed change in pH was used to calculate an instantaneous measurement of plasma buffer value in mM H^+ pH $unit^{-1}$ L^{-1} at pH 8.

2.1.5 Carbonic anhydrase characteristics in chondrichthyan gills

A subcellular fractionation protocol was conducted on gill tissue from 4 chondrichthyan and 2 teleost species according to the procedures of Henry et al. (1986, 1988, 1993, 1997). 1g of gill tissue was added to 2ml of ΔpH assay buffer then homogenized using a Teflon/glass homogenizer with 5 passes of 10 seconds (power = 7) until all visible pieces of tissue were gone. The homogenate was added to 6ml more buffer and then subjected to a series of centrifugation steps. The first was centrifugation of the crude homogenate at 8,500 g for 20 min at 4˚C to separate mitochondria and cell debris (pellet 2) from cytoplasm and microsomes (supernatant 2). Supernatant 2 was then centrifuged at 100,000 g for 90 min at 4˚C to separate microsomes (pellet 3) from cytoplasm (supernatant 3). Finally, pellet 3 was tested for CA activity then resuspended in ΔpH assay buffer, sonicated (5W 3s) and centrifuged at 100,000 g for 90 min at 4˚C to separate the true microsomal pellet (pellet 4) from any soluble CA that was not separated by the

first centrifugation (supernatant 4). This last step is referred to as the washing step and has been shown to significantly decrease the CA activity associated with the microsomal fraction. One washing step was deemed sufficient based on evidence from Esbaugh et al. (2004b) where additional washing steps did not affect CA activity of the microsomal fraction.

Pellet 4 was resuspended in buffer, sonicated again (5W 3S) and tested for CA activity. 700μL of the resuspended pellet was treated with one unit (10μL of 100 unit/mL) of phosphatidylinositol phospholipase C (PI-PLC), an enzyme that cleaves GPI linkages (Zhu and Sly, 1990; Gervais and Tufts, 1998; Gilmour et al., 2002). A control group was treated with 10μL of ΔpH assay buffer. Both the PI-PLC and control treatments were incubated for 90 minutes at 37˚C and centrifuged at 100,000 g for 90 minutes at 4˚C. The supernatant and resuspended pellet were tested again for CA activity and protein content. In addition, CA activity was measured in the resuspended pellet (P4) and cytoplasmic fractions (S3) before and after the addition of 0.005% sodium dodecyl sulphate (SDS), a detergent that inhibits soluble CA isoforms but has little effect on CAIV (Whitney and Briggle, 1982).

Histological staining was used to visualize gill structure and composition in each species. Samples were dehydrated in an ethanol series, cleared in xylene and infiltrated and embedded in paraffin wax. Paraffin sections (5μM thick) were mounted onto clean slides and de-waxed in xylene. All sections were treated with hematoxylin (stains nuclei, some granules and some calcified structures blue) and sections were also treated with one of three histological stains (Table 2.3).

For immunohistochemistry, samples were prepared according to the procedures outlined in Wilson et al. (2000) and Gilmour et al., (2007). Gill tissue was dehydrated, embedded and sectioned as above, but sections were mounted onto 3-aminopropyltriethoxysilane (APS; Sigma) coated slides and dried completely at 37˚C. Sections were de-waxed in xylene, tissue was circled

with a hydrophobic barrier pen (PAP pen, Sigma-Aldrich, Oakville, ON, Canada) and rehydrated with Bløk noise cancelling reagent (Sigma-Aldrich, Oakville, ON, Canada) for 20 minutes. Sections were then incubated with rabbit CAIV antibody (same as used by Gilmour et al., 2007), diluted 1:500) and rabbit anti-peptide Na^+/K^+-ATPase polyclonal antibody (Ura et al. 1996; Wilson et al. 2007; diluted 1:100) for 2hr at 37˚C. Slides were rinsed with TPBS (1% Tween-20 in phosphate buffered saline, pH 7.4) for 5, 10 and 15 minutes in Coplin jars then incubated with goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555 conjugated secondary antibodies (Fisher Scientific, 1:500). Following secondary incubation, slides were rinsed with TPBS for 5 minutes then nuclei were stained by incubating with DAPI for ten minutes before final 5 and 10 minute washes with TPBS. Slides were mounted with a glycerol mounting media (1:1 glycerol:TPBS with 0.1% sodium azide as preservative) and viewed on a Leica DM5500 (Concord, ON, Canada) epi-fluorescence microscope. Images were captured using a digital camera (Hamamatsu C11440, Bridgewater, NJ, USA). Plates were assembled in Adobe Photoshop CS6 and brightness and contrast adjusted while maintaining the integrity of the data.

2.1.6 Statistical analysis

All data are expressed as mean ± SEM and sample sizes are stated on all figures. Differences in CA activity and buffering capacity between species were compared by a one-way ANOVA with Tukey or Dunnett's post hoc tests. Endogenous plasma inhibitor and gill subcellular fractionation data were analyzed using t-tests (two sample or paired). A repeated measures ANOVA was used to test differences in CA activity with different amounts of CO_2-saturated water. For all tests, α=0.05 and where data deviated from the assumption of normality, log transformed values were used.

2.2 Results

2.2.1 Carbonic anhydrase characteristics in chondrichthyan blood

Validation of the ΔpH assay was conducted by measuring the activity of RBC CA when initiating the reaction with different volumes of CO_2-saturated water (Figure 2.1). In dogfish, 100µL of CO_2-saturated water used to initiate the ΔpH assay produced 79.8 ± 0.03% of the CA activity observed if 500µL CO_2-saturated water was added but there was no significant difference (Figure 2.1, repeated measures ANOVA with Tukey post-hoc tests, $p<0.05$) and consequently 100µL of CO_2-saturated water was used in all additional assays. The effect of perturbing solutes found in chondrichthyan blood was tested by measuring RBC and plasma CA activity in the presence of 340mmol L^{-1} urea and 70mmol L^{-1} TMAO. Urea and TMAO significantly decreased RBC CA activity by 12.5 ± 2.1% (paired t test $p=0.002$, $n=5$) but had no effect on plasma CA activity 15.0 ± 17.8% (paired t test $p=0.603$, $n=4$).

RBC CA activity was significantly different among species (Figure 2.2, $F=29.6$, $df=13$, $p<0.001$, one-way ANOVA on log transformed data with Tukey post-hoc tests). Rainbow trout (*Oncorhynchus mykiss*) had the highest activity of all species but interestingly, the RBC CA activity of rainbow trout was not significantly different from spotted ratfish (*Hydrolagus colliei*). Bigeye thresher sharks (*Alopias superciliosus*) had the lowest activity of the shark species and the lowest activity of any chondrichthyan was found in the ocellate river stingray (*Potamotrygon motoro*).

Plasma CA activity measurements were more variable than RBC values and plasma CA activity was only significantly different from rainbow trout in blue and shortfin mako sharks (Figure 2.3, $F=7.759$, $df=14$, $p<0.0001$, one-way ANOVA on log transformed data with Dunnett's post-hoc tests,). No evidence of an endogenous inhibitor was found in any

chondrichthyan species but significant inhibition was detected in rainbow trout (97.9 ± 0.7%) and coho salmon (89.0 ± 1.1%; Figure 2.4, paired t-test, $p<0.05$). Hemoglobin was detected at low levels in all plasma samples and plasma proteins ranged from 5.3 to 25.2 mg/mL in chondrichthyans and from 12.7-62.8 mg/mL in teleosts (Table 2.4).

2.2.2 Plasma buffer values

Pacific spiny dogfish had significantly higher plasma buffer values than blue sharks (*Prionace glauca*; Figure 2.5). Sample sizes in the rest of the species were too low to permit statistical tests from being performed.

2.2.3 Carbonic anhydrase characteristics in chondrichthyan gills

Carbonic anhydrase activity differed among species in the cytoplasm (Table 2.5; $F = 14.62$, $df = 5$, $p<0.001$), microsomes ($F = 29.42$, $df = 5$, $p<0.001$) and washed microsomal ($F = 107.3$, $df = 5$, $p<0.001$) fractions of gill tissue. Cytoplasmic CA activity, the activity within epithelial cells of the gills, was highest in rainbow trout and 59% and 65% higher than in coho salmon and Pacific spiny dogfish, respectively (Table 2.5). Blacktip shark (*Carcharhinus limbatus*), Atlantic stingray (*Dasyatis americana*) and ocellate river stingray cytoplasmic activity were significantly (78%, 76% and 83%, respectively) lower than dogfish. Microsomal CA activity was detected in all species and washing decreased the activity in most species but this relationship was only statistically significant in rainbow trout (Figure 2.6). Pacific spiny dogfish showed significantly higher washed microsomal pellet activity (183 ± 13.2 μmol CO_2 min^{-1} mg $protein^{-1}$) than all other species. Treatment with PI-PLC increased supernatant activity and pellet activity in all four chondrichthyans but not in teleosts (Figure 2.7). The extent of CA inhibition

after treatment with 0.005% SDS was significantly higher in cytoplasmic (45-70% of original value) than microsomal (75-95% of original value) fractions in 3 out of 4 chondrichthyans (two sample t-test, $p<0.05$; Figure 2.8). Inhibition by SDS in blacktip sharks was also higher in cytoplasmic fractions but this relationship was not statistically significant. The extent of inhibition between cytoplasmic and microsomal fractions was similar in teleosts (60-80% of original value; two sample t-test, $p>0.05$).

Histological images show the general structure of the gills in teleost (Figure 2.9A and B) and chondrichthyan (Figure 2.9C-F) gills. Red blood cells are visible in sections stained with eosin, indicating some red blood cell contamination in perfused gill tissue. Goblet cells are visible in the second and third panels of all species. The second and third panels also show large amounts of staining that appears to be uniform across the tissue and this is likely due to the staining of connective tissues, polysaccharides and basal lamina.

Immunohistochemical images show the distribution of CA, NKA and cell nuclei (Figures 2.10-2.15). Blood spaces are easier to observe in Pacific spiny dogfish and blacktip shark than in all other species, possibly due to the orientation of tissue during paraffin embedding. The lining of pillar cells was stained most brightly in Pacific spiny dogfish (Figure 2.11) whereas rainbow trout and coho salmon showed no visible CA staining on pillar cells (Figure 2.10, 2.11). Blacktip shark, Atlantic stingray and ocellate river stingray gills showed CA staining, but less brightly around the pillar cells than Pacific spiny dogfish (Figure 2.12-2.15). Atlantic stingray gills stained particularly strongly for CA throughout the tissue. Gills from rainbow trout and coho salmon stained for CA in goblet cells.

2.3 Discussion

This study aimed to determine whether the dogfish model of CO_2 excretion could be applied more generally to all chondrichthyan fishes. In general, data for the chondrichthyan species examined in this study supported a role for both the plasma and RBC in CO_2 excretion because of their lower RBC CA activity than rainbow trout, circulating plasma CA activity, lack of endogenous plasma CA inhibitor, higher plasma buffer values and membrane-bound type IV-like CA at the gills. However, there were more differences between species than expected. There were significant differences in RBC CA activity between chondrichthyan species and overall, activity varied by almost 300 μmol CO_2 min^{-1} mg $protein^{-1}$. Significant circulating plasma CA activity was only found in two chondrichthyan species. Plasma buffer values appeared higher than teleost values, but estimates for some species were much higher than others. While type IV-like CA was found at the gills of all chondrichthyan species, the gills of Pacific spiny dogfish had 4-5x more CA activity in washed microsomal fractions than any other species (Figure 2.5). In general, the dogfish model of CO_2 excretion does seem to apply to chondrichthyan fishes, but several notable exceptions exist that will be elaborated upon below.

2.3.1 Carbonic anhydrase characteristics of chondrichthyan blood

Consistent with previous findings, rainbow trout had higher RBC CA activity than Pacific spiny dogfish (Figure 2.2, Henry et al., 1997; Esbaugh et al., 2004) and most other chondrichthyans showed similar activity to Pacific spiny dogfish. Unexpectedly, the CA activity of rainbow trout RBCs was not significantly different than that of spotted ratfish. Based on studies from teleost fishes, the proximate cause of higher RBC CA activity is differences in CA concentration, rather than in the enzyme's kinetic properties (Esbaugh et al., 2004). This idea is

reinforced by the discovery that the amino acid substitution in the proton shuttling ligand of dogfish RBC CA is also found in seven other species of sharks and rays (Angelina Dichiera, *unpublished data*), suggesting that the same low activity isoform is present in all chondrichthyans. The ultimate explanation of differences in activity is not as well understood. Larimer and Schmidt-Nielsen (1960) found a relationship with RBC CA activity and body size in mammals, but no significant relationship was found in chondrichthyans (Appendix A1). In this study, bigeye thresher sharks and ocellate river stingrays had lower relative RBC CA activity than most other species (Figure 2.2). One hypothesis for the functional significance of low RBC CA activity was that it provided an advantage for animals in hypoxic conditions (Yang et al., 1998, 2000) which bigeye thresher sharks encounter on their diel vertical migrations up to 1000m (Weng and Block, 2004), and ocellate river stingrays may encounter when burrowing into sandy substrates. It was hypothesized that an acidosis due to low activity CA would cause an increased oxygen uptake by stimulating a higher breathing rate or increase oxygen delivery by right-shifting the oxygen equilibrium curve (Yang et al., 1998). If this were true, however, this study would have also found low RBC CA activity in blue and shortfin mako sharks which also make vertical migrations up to 980 and 620m, respectively (Stevens et al., 2010), and southern stingrays (*Dasyatis americana*) which also burrow into sandy substrates. Since these other species do not have similarly low RBC CA activity, it is unlikely that hypoxia tolerance plays a role in RBC CA activity in this study. Overall, the data suggest that chondrichthyan species show a wide range in RBC CA activity that overlaps with some teleost species. Teleosts also show a range of RBC CA activity (Esbaugh et al., 2004) and in fact, rainbow trout have the highest activity of all species examined, indicating that they may not be a representative teleost to compare with other vertebrate groups. Nevertheless, the results of this study were consistent with the proposed chondrichthyan model of CO_2 excretion, but since RBC CA is not limiting in any

species that has been examined (see Swenson, 2000), other measurements like endogenous plasma inhibitors may be more informative when examining CO_2 excretion strategies.

Teleost RBC CA activity was almost completely inhibited by the addition of 100µl plasma, but no evidence of inhibition was found in any chondrichthyan species tested (Figure 2.4). Despite its presence in teleosts and some mammals, the function of endogenous plasma CA inhibitors is still unclear. One hypothesis is that the primary function is in zinc recycling and the inhibitory properties are secondary (Henry and Heming, 1998). Since little is known about the inhibition and physical properties of endogenous plasma CA inhibitors, more studies are needed to uncover their true function. In this study, not only was there no evidence of any inhibitor, but there was also negative inhibition present in most species. This is partly due to the presence of detectable plasma CA activity in all chondrichthyan species, but is unlikely to account for the extent of the negative inhibition. Presence of even low concentrations of Hb (10µM) have been shown to double the rate of CA activity from bovine and human red cells (Silverman et al., 1979; Backman, 1981). So, not only can the presence of Hb indicate CA contamination from RBC lysis, Hb itself may bind CA, enhancing the efficacy of proton transfer (Silverman et al., 1979). The negative inhibition observed could be a result of Hb from plasma samples enhancing the activity of RBC and plasma CA. Nevertheless, no evidence of an endogenous plasma CA inhibitor was found in any chondrichthyan species which is consistent with the proposed chondrichthyan model of CO_2 excretion and suggests that there may be detectable CA activity in the plasma.

Among the 13 species tested in this study, CA was detectable in all plasma samples but was only significantly higher than rainbow trout in blue and shortfin mako sharks (Figure 2.3). Measurements of plasma CA activity in dogfish were higher than some previous estimates (4.06 µmol CO_2 min^{-1} ml^{-1}; Gilmour et al., 1997) and similar to others (~10-15 µmol CO_2 min^{-1} ml^{-1}; Henry et al., 1997). Circulating plasma CA activity was originally thought to allow HCO_3^-

dehydration at the gills to bypass Cl^-/HCO_3^- exchange, the rate-limiting step of CO_2 excretion (Wood et al., 1994). It was also thought to explain the efficient CO_2 excretion in dogfish (Pa_{CO2} ~ 1Torr, Ca_{CO2} <3mmol L^{-1}) despite their lack of a Haldane effect and lower HCO_3^- dehydration rate (Wood et al., 1994). However, Henry et al., (1997) calculated that circulating plasma activity in Pacific spiny dogfish accounts for only 0.1% of total blood CA and suggests that this is too little to contribute to CO_2 excretion. All other species in this study appeared to have higher plasma CA activity than dogfish but plasma CA still accounted for only 0.16-4.8% of whole blood CA activity when assuming a haematocrit of 20%. While this is higher than the estimates of Henry et al. (1997), it is likely that <5% of total CA activity is still insufficient for CO_2 excretion. Furthermore, the fact that most species showed detectable circulating plasma CA activity but not significantly higher activity than rainbow trout emphasizes the small role of circulating plasma CA activity in chondrichthyans. The exact source or function of circulating CA activity is still unknown, but it is thought to originate from normal RBC lysis and is only detectable in the cytoplasm due to the absence of a RBC inhibitor (reviewed in Henry and Heming, 1998). Similar to Henry et al. (1997), variability in circulating plasma CA was high within species in this study, so low levels of haemolysis cannot be excluded as a source of CA activity. As a proxy for RBC lysis, hemoglobin was measured in all plasma samples, and concentrations ranged from 1.3 to 16% of total blood hemoglobin levels normally seen in chondrichthyans (Table 2.4; percentages calculated using 1mmol L^{-1}; Phill Morrison, *unpublished data*). However, no relationship was observed between plasma CA activity and hemoglobin concentration (Figure A2). Additionally, plasma samples that comprised the four highest hemoglobin measurements by far (spotted ratfish, sandbar shark, cownose ray and Atlantic stingray) appeared cloudy but not at all pink when mixed with Drabkin's solution, indicating a confounding compound present that may have increased apparent hemoglobin

concentrations. Still, there were detectable levels of circulating plasma CA in all chondrichthyan species examined, again consistent with the proposed chondrichthyan model of CO_2 excretion.

2.3.2 Plasma non-bicarbonate buffer values

Plasma buffer values appeared higher for most chondrichthyans which is consistent with the chondrichthyan model of CO_2 excretion (Figure 2.5). Values for blue sharks, bigeye thresher sharks, cownose ray (*Rhinoptera bonasus*)and Xingu river stingrays were similar to plasma buffer values in dogfish (-6.5 mmol H^+ pH unit L^{-1}; Lenfant and Johansen, 1966) and *Raja ocellata* (-6.6 mmol H^+ pH unit L^{-1}; Graham et al., 1990) but higher than estimates from longnose skate (*Raja rhina*) and spotted ratfish (~-3 mmol H^+ pH unit L^{-1}; Gilmour et al., 2002). Despite the low values in longnose skate and spotted ratfish, the plasma still accounted for 40-60% of whole blood buffering (Gilmour et al., 2002). However, the buffer value of brown bullhead (*Ameiurus nebulosus*) plasma is also high (-5.7 mmol H^+ pH unit L^{-1}; Szebedinszky and Gilmour, 2002) and although it only accounts for 37% of whole blood buffering, this exception suggests that there are differences in absolute plasma buffer capacity within teleost fishes, as found for RBC CA activity (Esbaugh et al., 2004). Brown bullhead also lack an endogenous plasma CA inhibitor but have no branchial CAIV, making them an interesting species to further investigate CO_2 excretion strategies (Gilmour et al., 2002). Overall, these data provide further evidence that the buffer value of the plasma is not limiting to CO_2 excretion in a wide range of species and that if there is CAIV at the gills of all chondrichthyans, CO_2 excretion is not likely limited by proton availability.

2.3.3 CA characteristics of chondrichthyan gills

CA activity has been detected at the gills of all fish species examined, but the majority is cytoplasmic and hydrates CO_2 within epithelial cells to provide counter ions for ionoregulation (Henry et al., 1988; 1993; 1997; Sender et al., 1999; Gilmour et al., 2002). The present study found cytoplasmic CA activity in all species examined that was higher than microsomal CA activity, except in ocellate river stingray where the activities were approximately equal (Table 2.5, Figure 2.6). Comparatively, microsomal CA activity normally accounts for 0.2-5% of branchial CA activity but is important in its potential to catalyze plasma HCO_3^- dehydration (Henry et al., 1988; 1993; 1997; Sender et al., 1999; Gilmour et al., 2002). After washing, microsomal CA activity was highest in dogfish and all other species had less than 25% of dogfish activity. Treatment with PI-PLC decreased pellet and increased supernatant CA activity in all chondrichthyan species, indicating a release of GPI-linked CA from the membrane (Figure 2.7). In addition, CAIV is stabilized by disulphide bonds making it resistant to the detergent SDS, whereas the activity of cytoplasmic isoforms is negatively affected (Whitney and Briggle, 1982; Waheed et al., 1996). Microsomal CA activity from all chondrichthyan gills was resistant to SDS, again consistent with the proposed chondrichthyan model of CO_2 excretion where type IV CA is present at the gills (Figure 2.8). Microsomal CA activity was detected in rainbow trout and coho salmon, but this activity was neither susceptible to treatment with PI-PLC nor resistant to SDS, providing further support that CAIV is lacking in teleost gills. Immunohistochemical images confirm the presence of CA near the lining of pillar cells in dogfish (Figure 2.11), but the staining was less clear in the other three species (Figures 16-18). This discrepancy could have resulted from incompatibility of primary antibody sequences with CAIV sequences from these species and it would be best to make species-specific antibodies to confirm the lining of CAIV in pillar cells of blacktip sharks, Atlantic stingrays and ocellate river stingrays. The antibody also stained

for CA where cytoplasmic isoforms would be found, indicating that it is not specific to type IV CA. Nevertheless, it appears that CAIV is present at the gills of chondrichthyan fishes. When the results of this study are combined with the fact that hagfish also have CAIV at the gills (Esbaugh et al., 2009), they suggest that this may be an ancestral condition that has been retained in most vertebrate groups, including air-breathers (Whitney and Briggle, 1982; Waheed et al., 1992; Stabenau and Heming, 2003). Although there is strong evidence for type IV-like CA in all species examined in this study, whether this pool of CA activity contributes to CO_2 excretion remains to be seen. Biochemical assays and subcellular localization are useful techniques for examining the distribution and activity of CA, but in order to uncover its physiological function, *in vivo* experiments on CO_2 excretion (with and without CA inhibitors) in chondrichthyan species need to be conducted.

2.3.4 Summary

The results of this study suggest that the dogfish model of CO_2 excretion applies to all chondrichthyan fishes and contributes to our growing knowledge of the evolution of gas exchange and acid base balance in a basal vertebrate group. In general, the data show lower RBC CA activity, absence of an endogenous CA inhibitor, presence of some circulating CA activity, higher non-bicarbonate plasma buffer values and presence of type IV-like CA at the gills. However, more differences than expected were found between species. Rainbow trout had much higher RBC CA activity than all other species, and dogfish had much higher type IV-like CA activity than all other species. While it is useful to think broadly about models of CO_2 excretion in chondrichthyans and teleosts, the results of this study indicate that relationship between species and vertebrate groups can be complex (see Chapter 3 Section 2). Instead of distinct teleost and chondrichthyan models, it may be useful to think of CO_2 excretion strategies from

different species as belonging to a continuum with rainbow trout on one end and dogfish on the other and most species falling somewhere in between. *In vivo* experiments should also be conducted to determine whether both the plasma and RBC compartments contribute to CO_2 excretion in all chondrichthyan fishes. Overall, the results of this study indicate that chondrichthyan fishes have the capacity to follow the same CO_2 excretion pathway as dogfish. This finding suggests that chondrichthyan fishes are more similar to hagfish and mammals in their distribution of CA at the respiratory surface and the loss of CAIV in the gills of teleosts appears to be an exception. Further research on the basis of this loss would provide valuable information on the functional significance of carbonic anhydrase across vertebrate groups over evolutionary time. Still, confirmation that chondrichthyan fishes in general have the capacity to utilize both the red blood cell and plasma compartments for CO_2 excretion provides important information about patterns of gas exchange and acid base balance in this basal vertebrate group.

3 General discussion and conclusions

3.1 Thesis summary

This study provides the first evidence that the dogfish model of CO_2 excretion persists through the four main superorders of chondrichthyan fishes: Galeomorphii, Squalomorphii, Batoidea and Holocephali. According to this study, most chondrichthyan fishes have lower RBC CA activity, detectable circulating plasma CA activity, and lack an endogenous plasma CA inhibitor. It is important to note that a range of RBC CA activity exists in chondrichthyan fishes, similar to the range observed in teleosts, and the species with the highest RBC CA activity are not significantly different from some teleosts. Therefore, while this study supports separate teleost and chondrichthyan models of CO_2 excretion, the differences between the two groups may not be as pronounced as previously thought.

Higher plasma buffer values were also observed in most species, but further experiments using fresh plasma should be conducted to confirm this. Membrane-bound CA was also found in gill tissue from all chondrichthyan species examined and was released from the membrane by PI-PLC and resistant to SDS, indicating that it is CAIV. CA was also present in immunohistochemical images on the lining of pillar cells, although the localization of CA staining to the endothelial membrane was strongest in dogfish. Even after washing, CA was associated with the membrane fraction of teleosts, but this activity was neither susceptible to PI-PLC nor resistant to SDS, further supporting the idea that teleosts lack CAIV at the gills.

3.2 Perspectives on CO_2 excretion in vertebrate groups

Grouping species together can be useful when examining trends in CO_2 excretion characteristics because it allows us to think broadly about the reasons for one strategy over

another. For example, teleost fishes represent a large fraction of the total number of vertebrate species and many studies have examined their gas exchange characteristics. While this study has implied that chondrichthyans are an exception in fish CO_2 excretion, it is actually teleosts that are exceptional among vertebrates in this respect. Other vertebrate groups, even those that use only the RBC for CO_2 excretion, seem to have some CA source available to the plasma, but in teleosts this is uniformly absent. It is useful, then, in the discussion of the role of CA in gills and blood, to discuss why CA is absent from teleost plasma. Both prevailing theories are centred around teleost hemoglobin which has a pronounced Root effect (Root, 1931; Jensen and Weber, 1985; Brauner and Randall, 1996). One theory is that the presence of CA in the plasma short-circuits the protective mechanism for intracellular pH used by teleosts and compromises oxygen loading at the gills during a generalized acidosis (Rummer and Brauner, 2011; Rummer et al., 2013). However, this effect may not be large enough to compromise oxygen loading at the gills at lower concentrations of CA, indicating that a low level of CA in the plasma may not interfere with RBC pH regulation. Rather, the presence of CA in the plasma also decreases the cycling of HCO_3^- through the RBC and since HCO_3^- is needed to combine with protons released from hemoglobin, a decrease in HCO_3^- in the RBC may cause a pH change that does compromise O_2 transport (Lessard et al., 1995). Either way, the lack of CA activity available to teleost plasma is likely linked to the presence of a Root effect and associated red blood cell ß-NHE, traits that vary within teleosts but are absent from other vertebrate groups (Berenbrink et al., 2005; Randall et al., 2014). Perhaps this is why teleosts have evolved an endogenous plasma CA inhibitor and a lack of CAIV at the gills. However, some mammals also have a plasma CA inhibitor and the reason for its presence is largely unknown. Therefore, it is useful to combine species and think about CO_2 excretion strategies on a larger scale. However, broad scale approaches are not always representative of the true physiology of individual species and even though there are some traits

that are uniform within vertebrate groups, species-specific differences can complicate the broad picture of CO_2 excretion.

There are many species level differences in characteristics among vertebrates that are important to take into consideration when thinking about general models of CO_2 excretion. For example, this study was the first to show a wide range of type IV-like CA activity in the gills of chondrichthyans. While all species showed type IV-like CA at the gills, the activity varied among species and Pacific spiny dogfish had much higher activity than the other species. It is unclear whether gill CA activity is limiting to CO_2 excretion, but it is conceivable that less branchial CA activity results in a heavier reliance on the RBC for CO_2 excretion in some species. In addition, these species level differences are not restricted to chondrichthyans. For example, the presence and effectiveness of endogenous plasma inhibitors varies widely within teleosts. Inhibition ranges from 6% in channel catfish (*Ictalurus punctatus*) to 27% in lingcod (*Ophiodon elongates*) and 100% in rainbow trout (Henry et al., 1997). Inhibitors also appear to be species-specific because inhibition decreases markedly when a potent inhibitor from one species is used on another (Henry et al., 1997). Buffer value is another trait that varies within groups. Plasma buffer values in teleosts are generally low, but buffer values in brown bullhead are similar to that of Pacific spiny dogfish (Szebedinszky and Gilmour, 2002). The functional significance of differences in CA activity or buffer values within vertebrate groups is not well understood, but the differences themselves highlight that a broad approach to studying CO_2 excretion does not capture the diversity within groups. While it is notable that this study found the capacity for a similar CO_2 excretion strategy in such a diverse group as chondrichthyans, the differences between species highlight the importance of considering individual species within the context of larger vertebrate groups. The results of this study support a wide range for most characteristics within groups, and

suggest that a species-specific approach may be more effective in studying the specific roles of CA in different species and under different conditions.

Further complicating the comparison of major vertebrate groups, each group has a specific suite of physiological adaptations that have allowed them to be successful in their environments. One such adaptation is the use of urea as an osmolyte in the blood of chondrichthyan fishes. In this study, RBC and plasma CA activity were decreased by the addition of physiologically relevant concentrations of urea and TMAO, although only significantly in RBCs. Although it is less toxic than ammonia, urea is known to be a potent destabilizer of proteins by interfering with hydrophobic interactions and hydrogen bonds and can increase the K_m or decrease the V_{max} of enzymes (Yancey, 1985). The incorporation of TMAO as an osmolyte counteracts some of these detrimental effects, decreasing the K_m and increasing the V_{max} of affected enzymes to near normal levels (Yancey, 1985). Thus, when urea and TMAO are found together in a 2:1 ratio as osmolytes, they are compatible solutes, counteracting the negative effects of one another (Yancey, 1985). The effect of urea and TMAO on RBC activity in dogfish suggests that previous measurements in chondrichthyans may overestimate the true activity and underestimate the difference between teleost and chondrichthyan RBC CA activity. The case of urea and TMAO is only one example of a major difference between vertebrate groups that complicates the comparison of CO_2 excretion models between them.

3.3 CA and ionoregulation

Since H^+ and HCO_3^- are exchanged across membranes for Na^+ and Cl^-, respectively, the distribution of carbonic anhydrase at the gills is linked with ion regulation. Interestingly, there is also evidence of plasticity in branchial CA in some species. For example, CA shows a plastic

response to salinity in the European green crab (*Carcinus maenus*) where acclimation to 10ppt water causes an induction of CA activity in the posterior gills known for ion transport (Henry et al., 2003). This increase occurs mainly in the cytoplasm of epithelial cells, but also in membrane-bound CA, and presumably functions in the uptake of ions as they become limiting in the surrounding environment (Henry et al., 2003). Although CA is one of the fastest enzymes known and is not thought to be limiting to gas exchange in the RBC, an increase in gill CA activity seems to provide an advantage in low salinity, indicating that it may be limiting in this instance. The present study showed evidence for cytoplasmic and type IV-like CA at the gills of the euryhaline Atlantic stingray that migrates into rivers and lakes to complete its life cycle (Johnson and Snelson, 1996; Piermarini and Evans, 1998). To accomplish this, Atlantic stingrays decrease Na^+, Cl^+ and urea concentrations in the plasma, although urea is still 150-200mmol L^{-1} (Piermarini and Evans, 1998). However, even with a decrease in plasma ions, the diffusion gradient would be strong and individuals would need to actively take up Na^+ and Cl^-, and an increase in CA activity could facilitate this. The Atlantic stingrays in this study were captured in seawater, so the low membrane-bound activity compared to dogfish could be an underestimate of the capacity for CA at the gills during an ionoregulatory challenge. Therefore, it would be interesting to examine the response of CA to reduced salinity in this species, both at the gill and rectal gland. The involvement of CA in ionoregulation could explain the higher activity found in the gills of the stenohaline freshwater ocellate river stingray, but does not explain the much higher activity found in dogfish. Overall, the idea that gill CA may be limiting in certain situations and shows plasticity with environmental conditions presents an exciting new research direction, particularly in the face of global environmental change.

3.5 Research limitations and future directions

In vitro measurements like the ones in this study provide useful first steps towards uncovering the function of carbonic anhydrase in the gills and blood in chondrichthyan fishes. The main limitation of these studies is that the situation in a live animal can be quite different than the ones simulated *in vitro* and that is why *in vivo* measurements are necessary to confirm function. For example, RBC CA activity in this study was measured in a simple, diluted system whereas in an animal it acts in a complex, diffusion limited and dynamic intracellular environment (Gilmour, 2010). The electrometric ΔpH assay is run at 4˚C which is necessary to slow down CA enough to measure its rate, but is different than the temperatures that the ectothermic or regionally heterothermic animals in this study inhabit. In addition, preferred environmental temperatures for the species from this study range from approximately 10 to 25˚C, so CA activities could be increased to a different extent in the body of each species. While it would make ecological interpretations easier, measuring the action of CA at physiological temperatures would make CA activity measurements difficult to interpret. So, while *in vitro* measurements are useful and necessary, the ultimate goal should be to conduct *in vivo* experiments.

We now know that type IV-like CA exists in several chondrichthyan species, but its mere presence in subcellular localization studies is not enough to infer physiological function. Given the extent to which dogfish have been studied in this regard (Swenson and Maren, 1987; Gilmour et al., 1997; Wilson et al., 2000, Gilmour et al., 2001, 2004, 2007), it is likely that this pool of CA activity contributes to CO_2 excretion in other chondrichthyans, but it is not known for sure. To examine this, one could create an extracorporeal blood circulation by implanting two cannulae into the celiac artery, one in either direction (see Gilmour et al., 2001). These cannulae can be connected to each other and connected in series to pH, P_{CO2} and P_{O2} electrodes for continuous

blood measurements as described in Gilmour et al. (1994). The contribution of CAIV to CO_2 excretion could then be assessed through the measurement of respiratory variables after bolus injections of CA inhibitors to inhibit only extracellular CA (e.g. benzolamide, F3500) or all CA activity (e.g. acetazolamide). It would be important to test the efficacy of these inhibitors on each species before the experiment to ensure the proper CA pool is sufficiently inhibited. These experiments can be repeated under conditions of increased CO_2 loading by injection of HCO_3^- or the Cl^-/HCO_3^- exchanger inhibitor 4,4'-diisothiocyanostilbene-2-2'-disulphonic acid (DIDS). If extracellular CAIV at the gills was contributing to CO_2 excretion, the inhibition of CA should cause an acidosis in postbranchial blood due to impaired CO_2 excretion and an associated increase in PCO_2. It is also possible that an injection of PI-PLC could be used to wash CAIV out of the gills so that the contribution of circulating CA activity could be examined. One could also use the same cannulation protocol in addition to a caudal vein cannula that would be used to withdraw blood and replace it with saline to create anemic conditions while measuring pH, P_{CO2}, and P_{O2}. Overall, it will be necessary to make *in vivo* measurements to determine whether the type IV-like CA found at the gills of blacktip sharks, Atlantic stingrays and ocellate river stingrays contributes significantly to CO_2 excretion. However, in absence of such studies, measurements like plasma buffer values can provide insight into whether the correct physiological environment exists for plasma HCO_3^- dehydration.

The method for measuring plasma buffer values used in this study reduces the need for traditional tonometry methods that require large volumes of fresh plasma, but high-quality plasma was still required for this method to produce reliable results. For example, it was noted that if plasma samples contained any particulate matter, the buffer curves obtained would have larger variation and a higher buffer value at pH 8 (see dogfish measurements in Figure A3). Still, this method uses only 200µL of plasma which is much less than that required for tonometry

methods and is therefore a useful measurement when large quantities of blood are not available. All other species produced curves that were repeatable (if n>1) and therefore if high-quality plasma is used, calculated buffer values are likely representative of true values. Additionally, the buffer curves of several species in this study showed trends that would not have been detected using traditional tonometry methods (Figure A3). For example, all ray species showed an increase and subsequent decrease in plasma buffer value around pH 8 that was not observed in shark species. This trend was most notable in southern stingrays (*Dasyatis americana*) where plasma buffer values increased over 3-fold near pH 8. Perhaps there is a protein present in the plasma of these species that confers a large proportion of plasma buffer values in this group. In general, this new method provides a new perspective on plasma buffer values and may provide additional information that is not possible with traditional measurements.

The results of this study provide important baseline measurements of CO_2 excretion characteristics in several chondrichthyan species. Although this thesis contributes to our knowledge of CO_2 excretion strategies in vertebrates, *in vivo* experiments are necessary and I genuinely hope that this research is conducted by comparative physiologists in the future. Once we know how chondrichthyans excrete CO_2 under normal conditions, it will become important to examine if and how gas exchange and acid-base balance will be affected by changing environmental conditions.

Figures and tables

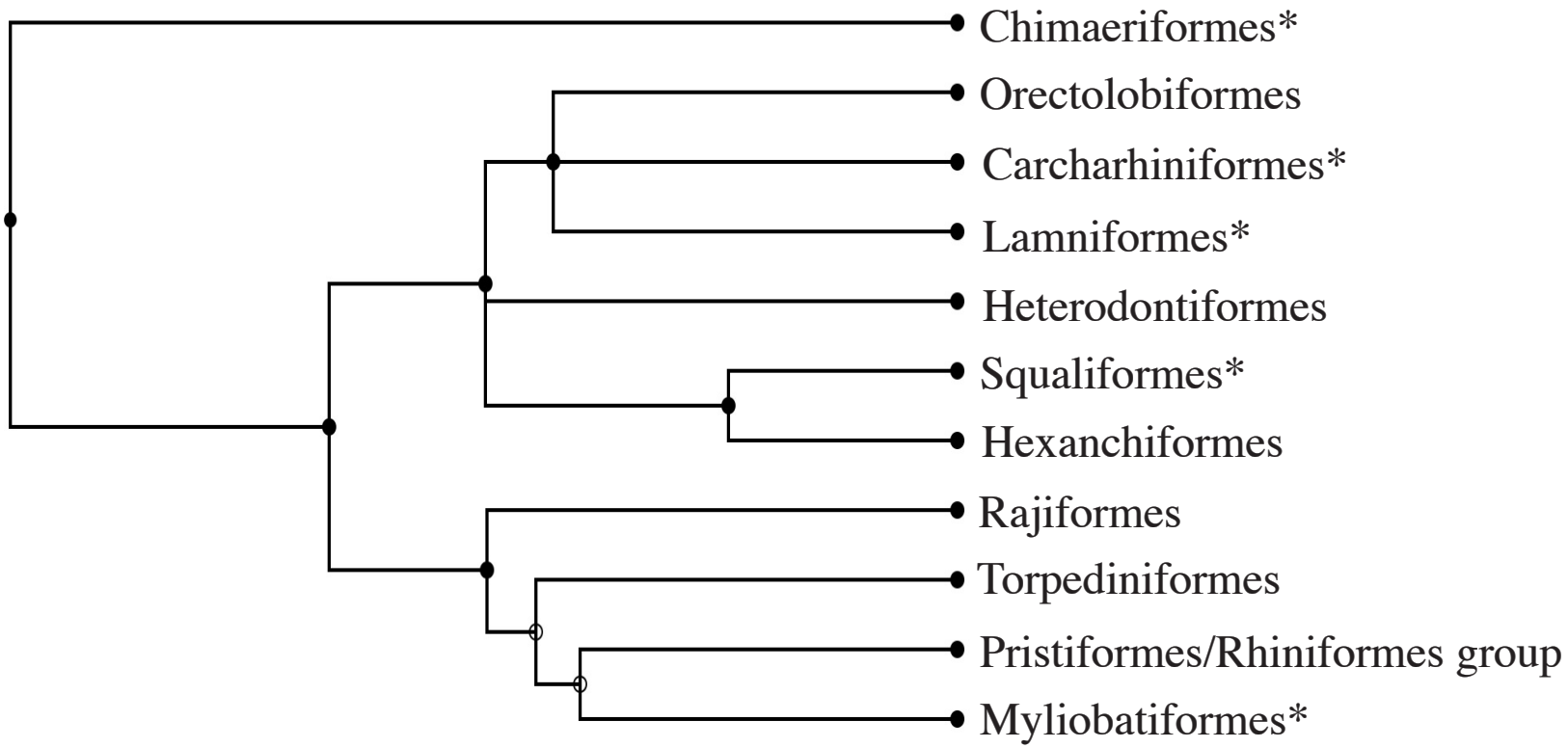


Figure 1.1 Phylogenetic tree of the existing orders of chondrichthyan fishes. Asterisks indicate orders for which samples were measured in this thesis.

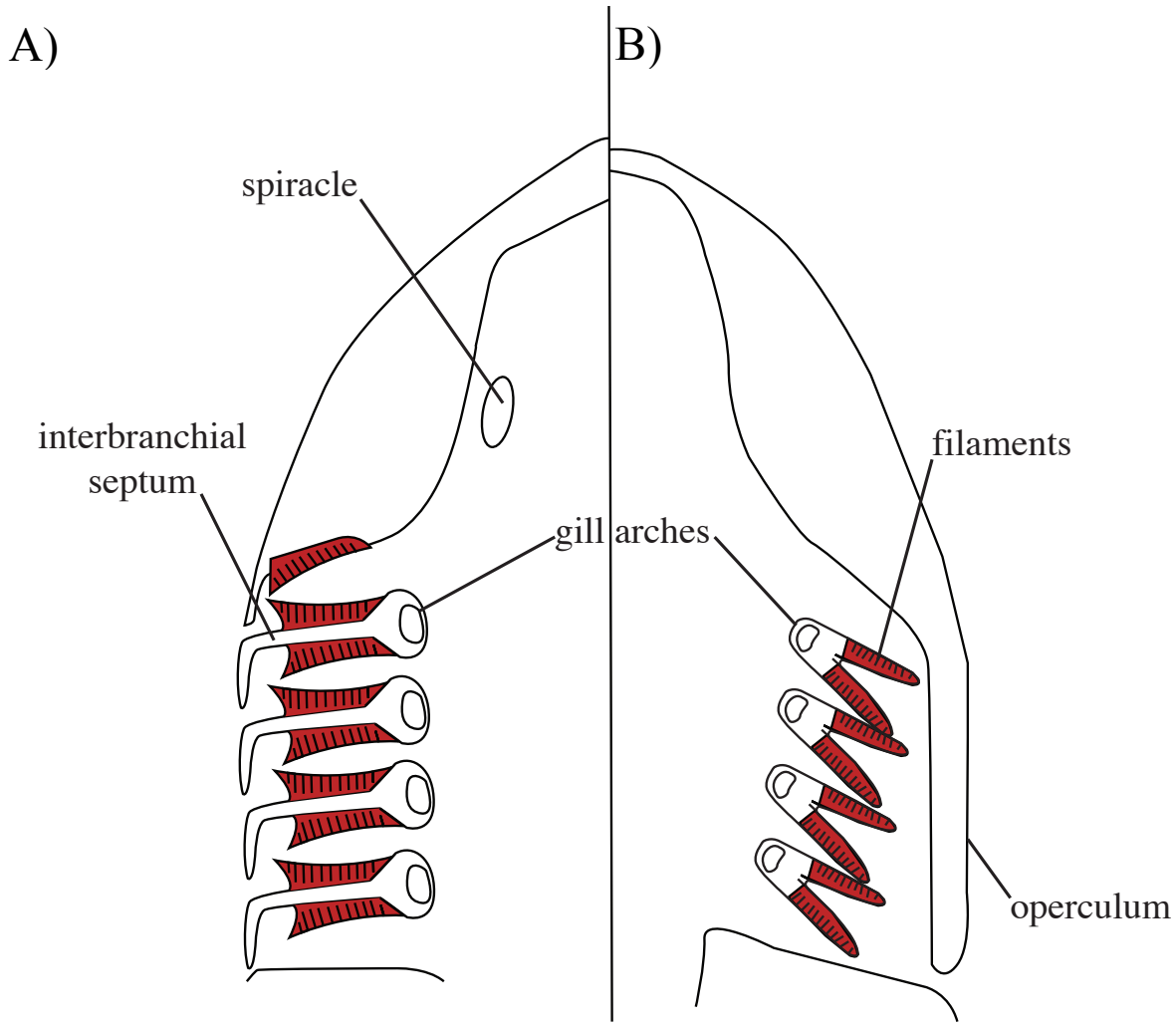


Figure 1.2. Differences in the general structure of A) chondrichthyan and B) teleost gills. Modified from Evans et al., (2005).

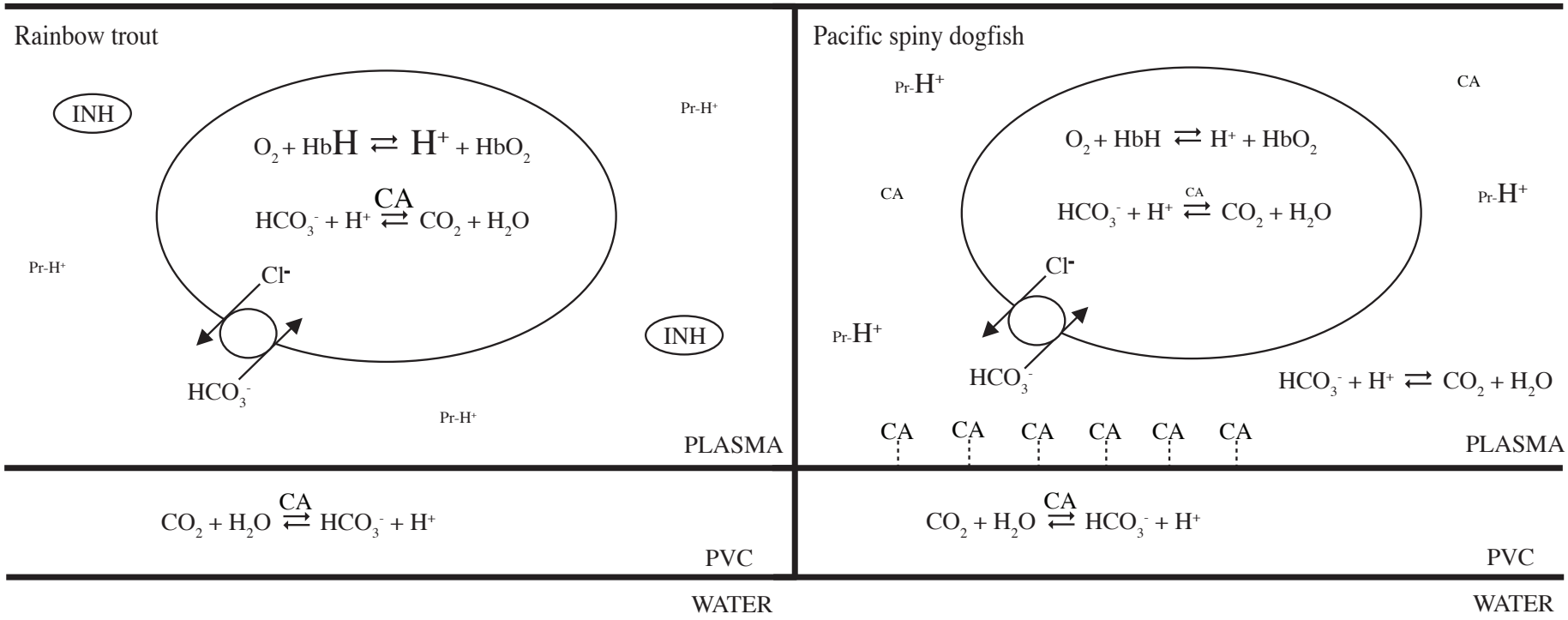


Figure 1.3. Characteristics of the teleost and chondrichthyan models of CO_2 excretion showing known characteristics in rainbow trout (*Oncorhynchus mykiss*) and Pacific spiny dogfish (*Squalus suckleyi*). CA = carbonic anhydrase, Hb = hemoglobin, INH = endogenous plasma CA inhibitor, Pr-H^+ = plasma non-bicarbonate buffer value, PVC = pavement cell.

Table 1.1. Comparison of teleost and dogfish models of CO_2 excretion based upon measurements in rainbow trout (*Oncorhynchus mykiss*) and the Pacific spiny dogfish (*Squalus suckleyi*). ß = non-bicarbonate buffer value, PI-PLC = phosphatidylinositol phospholipase-C, SDS = sodium dodecyl sulphate

	Rainbow trout (*Oncorhynchus mykiss*)	**Pacific spiny dogfish** (*Squalus suckleyi*)
RBC CA activity (mmol CO_2 min^{-1} ml RBC^{-1})	155[5]-513[1]	30.8[1]
Extracellular CA activity (µmol CO_2 min^{-1} ml^{-1})	0[1*]	4.06[1*]
% inhibition of RBC CA activity by plasma	100[1]	0[1]
Relative plasma ß (% whole blood value)	20-40[3]	40-70[3]
Gill microsomal CA activity (µmol CO_2 min^{-1} ml^{-1})	71 ± 35[2]	329 ± 28[2]
Sensitive to PI-PLC?	No[1,2]	Yes[1,2]
Sensitive to SDS?	Yes[4**]	No[2,4,6***]

[1]Henry et al., 1997; [2]Gilmour et al., 2001; [3]Gilmour et al., 2002; [4]Stabenau and Heming, 2003; [5]Esbaugh et al., 2005; [6]Gilmour et al., 2007
*corrected for uncatalyzed rate from Henry et al., 1997
**SDS concentration 0.2%, higher than concentration tested on chondrichthyans (0.005%)
***Not directly tested but inferred from confirmation of type IV CA[6] and by insensitivity shown in longnose skate *Raja rhina* (0.005% SDS)[2] and other species with CAIV in respiratory organs[4]

Table 2.1 List of chondrichthyan and teleost species for which blood and gill samples were obtained for this thesis. Taxonomic order, freshwater or marine origin and number of individuals for gill and blood samples from the supplying institution are also indicated.

	Species	Order	Freshwater or Marine?	Gills (n)	Blood (n)	Source
Teleosts						
	Rainbow trout (*Oncorhynchus mykiss*)	Salmoniformes	Freshwater	9	6	University of British Columbia, Vancouver, BC, Canada
	Coho salmon (*Oncorhynchus kisutch*)	Salmoniformes	Anadromous	3	3	University of British Columbia, Vancouver, BC, Canada
Chondrichthyans						
	Pacific spiny dogfish (*Squalus suckleyi*)	Squaliformes	Marine	5	5	Bamfield Marine Sciences Centre, Bamfield, BC, Canada
	Spotted ratfish (*Hydrolagus colliei*)	Chimaeriformes	Marine	0	4	Bamfield Marine Sciences Centre, Bamfield, BC, Canada
	Blacktip shark (*Carcharhinus limbatus*)	Carcharhiniformes	Marine	6	6	University of Texas Marine Science Institute, Port Aransas, TX, USA
	Blue shark *(Prionace glauca)*	Carcharhiniformes	Marine	0	8	Pfleger Institute for Environmental Research, Oceanside, CA, USA

Species	Order	Freshwater or Marine?	Gills (n)	Blood (n)	Source
Bigeye thresher shark (*Alopias superciliosus*)	Lamnidae	Marine	0	5	Dr. Chugey Sepulveda and Dr. Diego Bernal via Phil Morrison
Shortfin mako shark (*Isurus oxyrhinchus*)	Lamnidae	Marine	0	5	Dr. Chugey Sepulveda and Dr. Diego Bernal via Phil Morrison
Sandbar shark (*Carcharhinus plumbeus*)	Carcharhiniformes	Marine, Estuarine	0	8	Virginia Institute of Marine Science, Gloucester Point, VA, USA
Atlantic stingray (*Dasyatis sabina)*	Myliobatiformes	Euryhaline	5	5	University of Texas Marine Science Institute, Port Aransas, TX, USA
Southern stingray (*Dasyatis americanus*)	Myliobatiformes	Marine	0	2	Vancouver Aquarium, Vancouver, BC, Canada
Cownose ray (*Rhinopterus bonasus*)	Myliobatiformes	Marine	1	4	University of Texas Marine Science Institute, Port Aransas, TX, USA and Vancouver Aquarium, Vancouver, BC, Canada
Ocellate river stingray (*Potamotrygon motoro)*	Myliobatiformes	Freshwater	7	6	National Institute of Amazonian Research, Manaus, Amazonas, Brazil
Tiger stingray (*Potamotrygon tigrina)*	Myliobatiformes	Freshwater	0	3	Vancouver Aquarium, Vancouver, BC, Canada

Species	Order	Freshwater or Marine?	Gills (n)	Blood (n)	Source
Xingu river stingray (*Potamotrygon leopoldi)*	Myliobatiformes	Freshwater	0	3	Vancouver Aquarium, Vancouver, BC, Canada

Table 2.2. The three different fixation protocols used to fix gill tissue from chondrichthyan and teleost species. Rainbow trout (*Oncorhynchus mykiss*) were fixed using all three protocols (n=3 for each type) to test the effect of fixative on the immunohistochemical staining.

Fixation Protocol	Species
Immersion in 4% paraformaldehyde in phosphate-buffered saline followed by transfer to 70% ethanol for storage at 4˚C.	– Rainbow trout (*Oncorhynchus mykiss*) – Ocellate river stingray (*Potamotrygon motoro*) – Spotted ratfish (*Hydrolagus colliei*)
Immersion in 20% DMSO in 100% methanol at -20˚C for 48 hours followed by transfer to 100% methanol for storage at -20˚C.	– Rainbow trout (*Oncorhynchus mykiss*) – Pacific spiny dogfish (*Squalus suckleyi*) – Coho salmon (*Oncorhynchus kisutch*)
Immersion in Z-fix zinc formalin fixative (Anatech Ltd) for 48 hours followed by transfer to 70% ethanol for storage at 4˚C.	– Rainbow trout (*Oncorhynchus mykiss*) – Blacktip shark (*Carcharhinus limbatus*) – Spinner shark (*Carcharhinus brevipinna*) – Atlantic stingray (*Dasyatis sabina*) – Cownose ray (*Rhinopera bonasus*)

Table 2.3. Description of histological stains used on gill tissue from chondrichthyan and teleost species. All sections were stained with hematoxylin in addition to either eosin, periodic acid or alcian blue and periodic acid.

Stain	Colour	Structures stained
Eosin	Red	–intra- and extracellular proteins –most cell cytoplasms –RBCs
Peridoic acid	Purple	–polysaccharides –basal lamina –connective tissues –mucous
Alcian blue and peridoic acid	Bluish green	–mucus (including goblet cells) –polysaccharides

Table 2.4 Plasma sample hemoglobin (as an indication of hemolysis) and plasma protein concentrations of teleost and chondrichthyan species for which CA activity and/or non-bicarbonate buffer value were measured. Values are means ± SEM and sample sizes are shown in parentheses.

	Hemoglobin (mmol L^{-1})	**Plasma Protein (mg/mL)**
Teleosts		
Rainbow trout (*Oncorhynchus mykiss*)	0.0062 ± 0.001 (6)	62.8 ± 9.5 (6)
Coho salmon (*Oncorhynchus kistuch*)	0.0098 ± 0.003 (3)	12.17 ± 0.7 (3)
Chondrichthyans		
Spotted ratfish (*Hydrolagus colliei*)	0.093 ± 0.04 (4)	14.3 ± 1.5 (4)
Pacific spiny dogfish (*Squalus suckleyi*)	0.013 ± 0.002 (3)	9.1 ± 1.4 (4)
Bigeye thresher (*Alopias superciliosus*)	0.027 ± 0.003 (5)	11.0 ± 3.2 (5)
Sandbar shark (*Carcharhinus plumbeus*)	0.11 ± 0.006 (5)	25.2 ± 1.3 (5)
Shortfin mako shark (*Isurus oxyrhinchus*)	0.087 ± 0.04 (5)	18.7 ± 3.2 (5)
Blacktip shark (*Carcharhinus limbatus*)	0.038 ± 0.02 (4)	13.8 ± 1.7 (5)
Blue shark (*Prionace glauca*)	0.038 ± 0.01 (5)	10.9 ± 1.0 (3)
Cownose ray (*Rhinoptera bonasus*)	0.16 ± 0.04 (4)	12.9 ± 4.2 (3)
Southern stingray (*Dasyatis americana*)	0.054 ± 0.005 (2)	12.2 ± 0.5 (2)
Atlantic stingray (*Dasyatis sabina*)	0.12 ± 0.03 (5)	12.6 ± 1.1 (5)
Xingu river stingray (*Potamotrygon leopoldi*)	0.045 ± 0.05 (2)	6.9 ± 1.4 (3)
Tiger stingray (*Potamotrygon tigrina*)	0.036 ± 0.01 (3)	5.3 ± 0.9 (3)
Ocellate river stingray (*Potamotrygon motoro*).	0.039 ± 0.01 (5)	7.9 ± 1.3 (6)

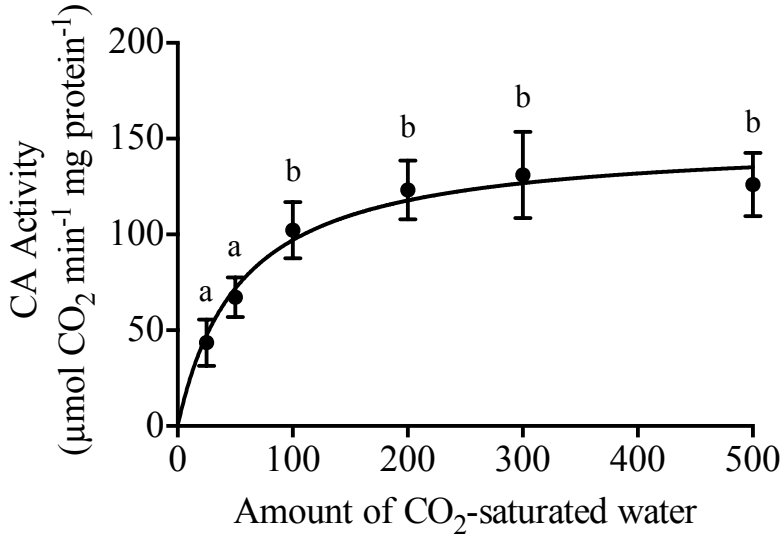


Figure 2.1. Effect of increasing amounts of CO_2-saturated water in the *in vitro* CA assay for measurement of RBC CA activity of Pacific spiny dogfish (*Squalus suckleyi,* n=5, mean ± SEM) with a Michaelis-Menten curve. Significant differences between points are indicated by lowercase letters (repeated measures ANOVA with Tukey post-hoc test, $P<0.05$).

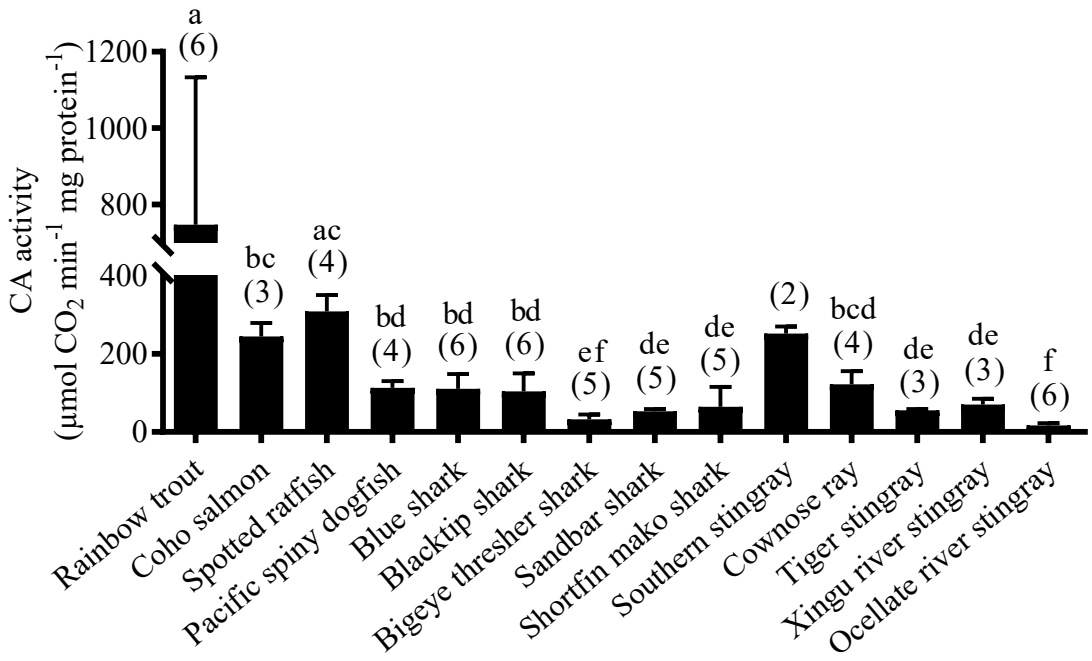


Figure 2.2. Red blood cell CA activity (mean ± SEM) from two teleost (rainbow trout and coho salmon) and twelve chondrichthyan species. Sample sizes are shown in parentheses and significant differences between species are indicated by letters that differ (one-way ANOVA with Tukey post-hoc tests, $p<0.05$).

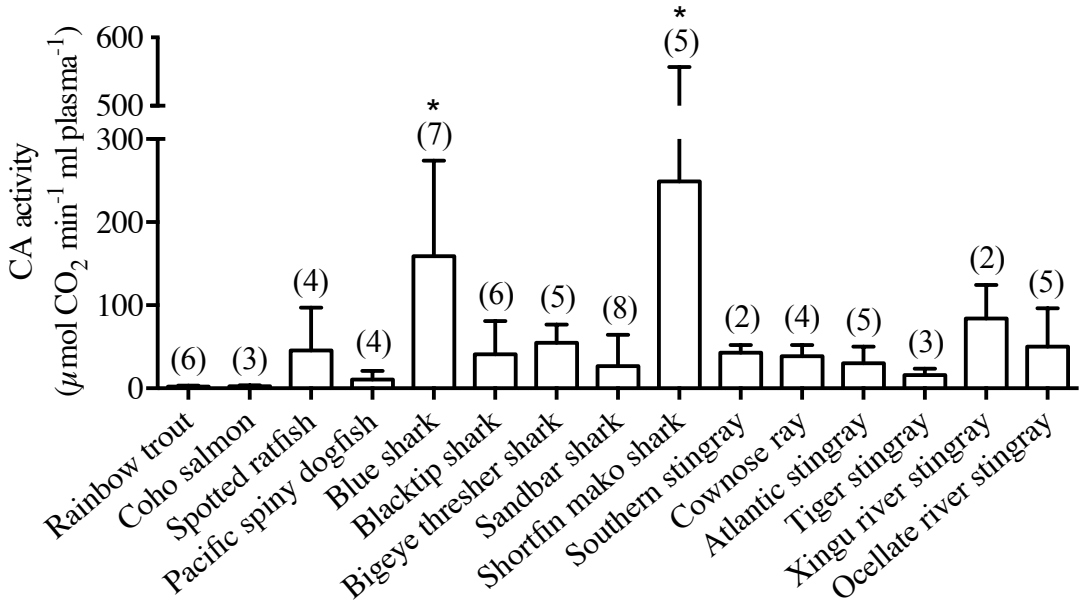


Figure 2.3. Extracellular CA activity (mean ± SEM) from two teleost and thirteen chondrichthyan species. Significant difference from rainbow trout is indicated by an asterisk (one-way ANOVA with Dunnett's post hoc test, $p<0.05$) and sample sizes are shown in parentheses.

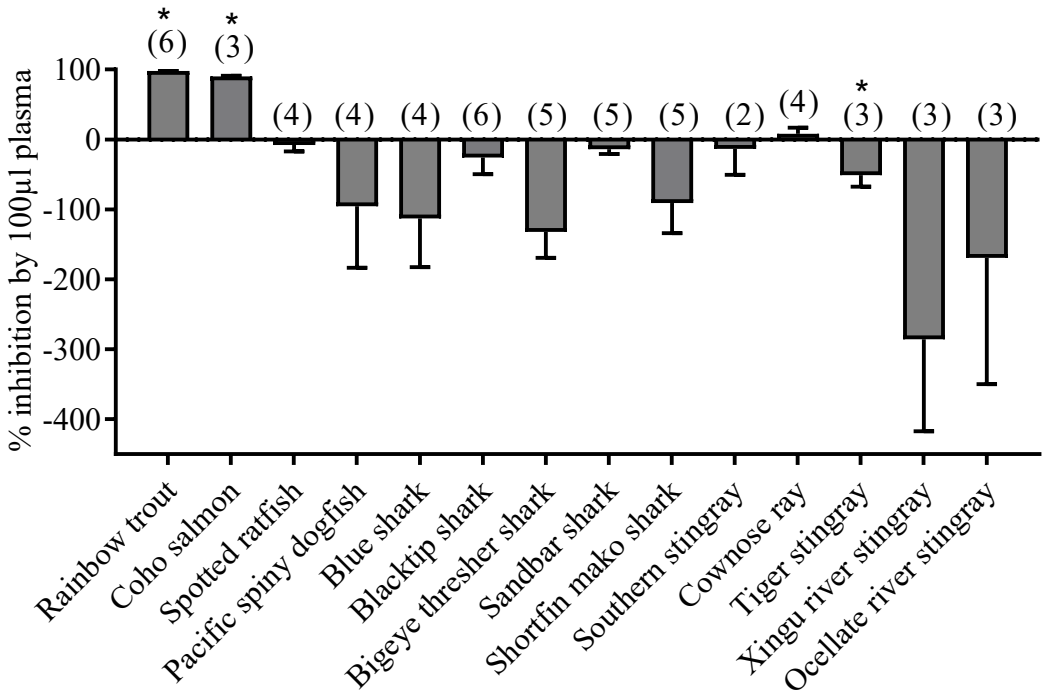


Figure 2.4. Percent (%) inhibition (mean ± SEM) of RBC CA activity following the addition of 100µl of plasma in two teleost and twelve chondrichthyan species. Asterisks indicate a significant change in RBC CA activity of a particular species after the addition of 100µL plasma. Plasma from the respective species was used for measurement of inhibition and sample sizes are shown in parentheses.

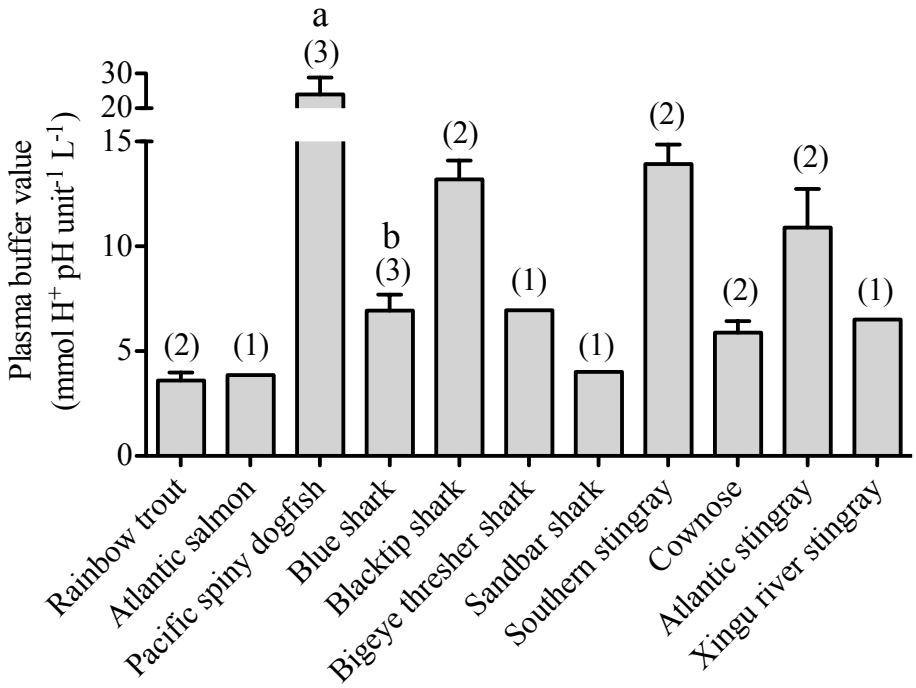


Figure 2.5. Buffer values of the plasma at pH 8 (using double endpoint titrations) conducted on 200µL plasma from each species. Teleost values are from data collected by Mike Sackville and different letters indicate significant differences between species (one-way ANOVA with Tukey post-hoc tests, $p<0.05$).

Table 2.5. CA activity (mean ± SEM) of the cytoplasmic fraction of gill tissue samples from two teleost and four chondrichthyan species. Sample sizes are shown in parentheses and lowercase letters denote significant difference between species as a result of a one-way ANOVA ($\alpha = 0.05$).

Species	**Cytoplasmic CA activity (μmol CO_2 min^{-1} ml^{-1})**	**Cytoplasmic CA activity (μmol CO_2 min^{-1} mg $protein^{-1}$)**
Teleosts		
Rainbow trout (*Oncorhynchus mykiss*)	1723.0 ± 363.3^{a} (6)	700.7 ± 142.6^{a} (6)
Coho salmon (*Oncorhynchus kisutch*)	1218.1 ± 121.2^{ab} (3)	286.1 ± 31.8^{b} (3)
Chondrichthyans		
Pacific spiny dogfish (*Squalus suckleyi*)	503.6 ± 38.1^{b} (5)	245.7 ± 8.5^{b} (5)
Blacktip shark (*Carcharhinus limbatus*)	156.4 ± 19.7^{c} (5)	55.3 ± 7.2^{c} (5)
Atlantic stingray (*Dasyatis sabina*)	141.7 ± 22.8^{cd} (5)	60.0 ± 5.4^{c} (5)
Ocellate river stingray (*Potamotrygon motoro*)	69.7 ± 4.8^{d} (6)	41.3 ± 2.7^{c} (6)

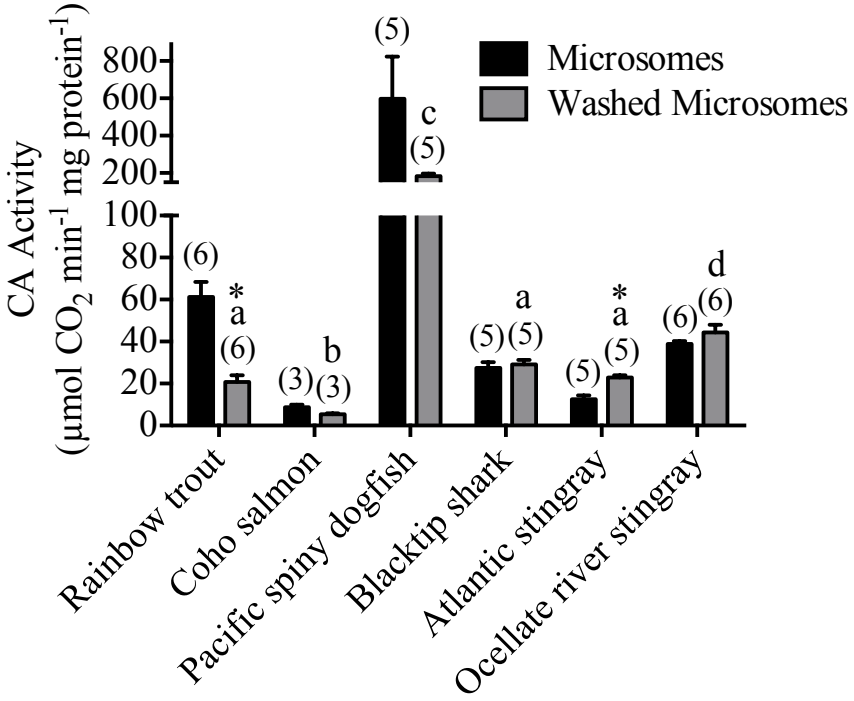


Figure 2.6. CA activity (mean ± SEM) associated with the microsomal fraction of gill tissue from two teleost and four chondrichthyan species both before (black bars) and after (grey bars) a washing step. Sample sizes are shown in parentheses, an asterisk denotes a significant difference before and after washing (paired t-test, $\alpha = 0.05$) and lowercase letters indicate a difference in washed microsome activity between species (one-way ANOVA $F = 107.3$, $df = 5$, $p<0.001$; Tukey post-hoc comparison).

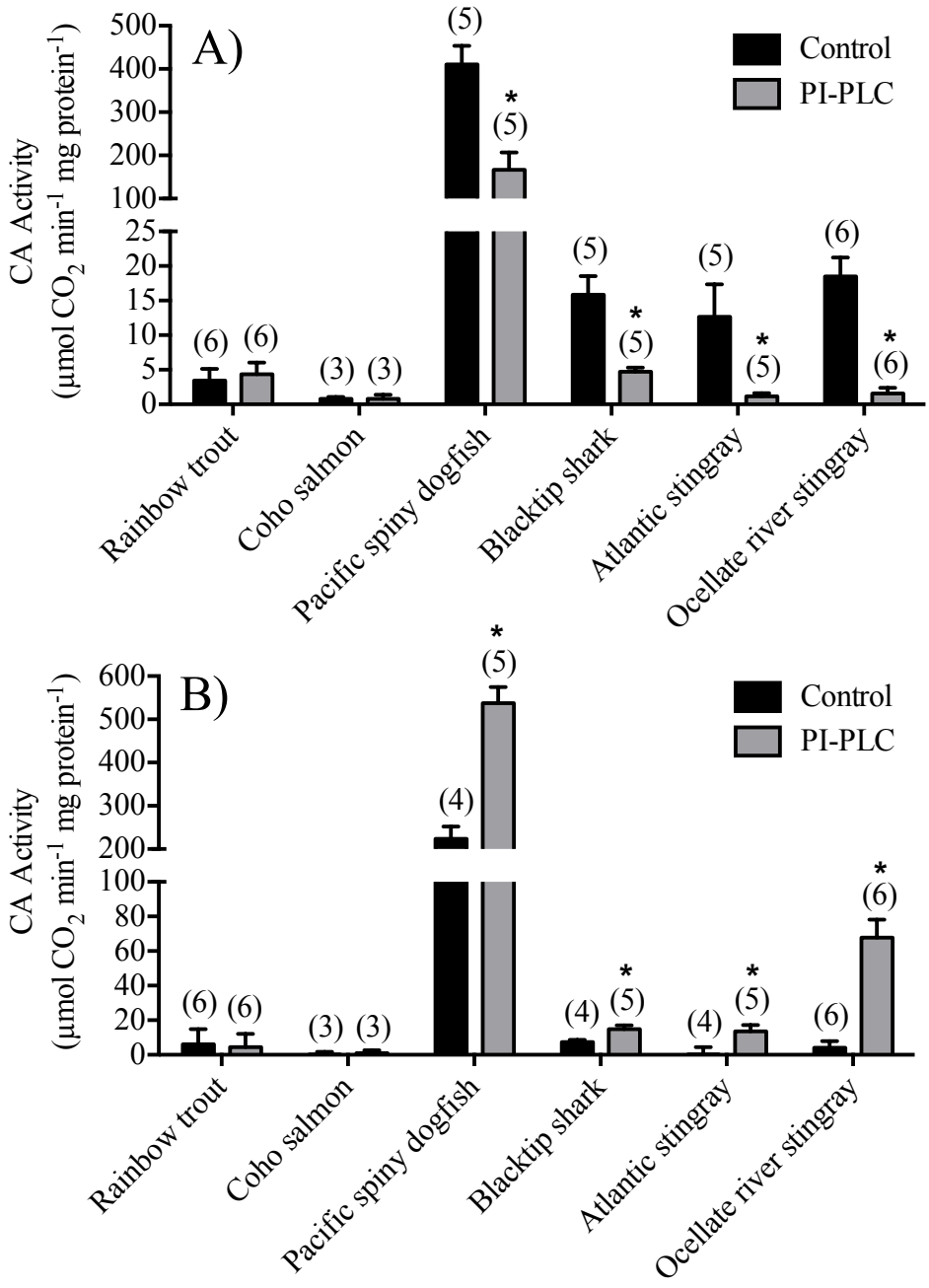


Figure 2.7. CA activity (mean ± SEM) associated with the A) pellet and B) supernatant fractions of gill tissue before after treatment with buffer (control; black bars) or one unit phosphatidylinositol phospholipase-C (PI-PLC; grey bars). Together, a significant decrease in pellet activity and increase in supernatant activity indicates a glycosylphosphatidylinositol-linked (GPI-linked) CA associated with the gill membrane. Sample sizes are shown in parentheses and an asterisk denotes a significant difference before and after treatment (two sample t test, $\alpha = 0.05$).

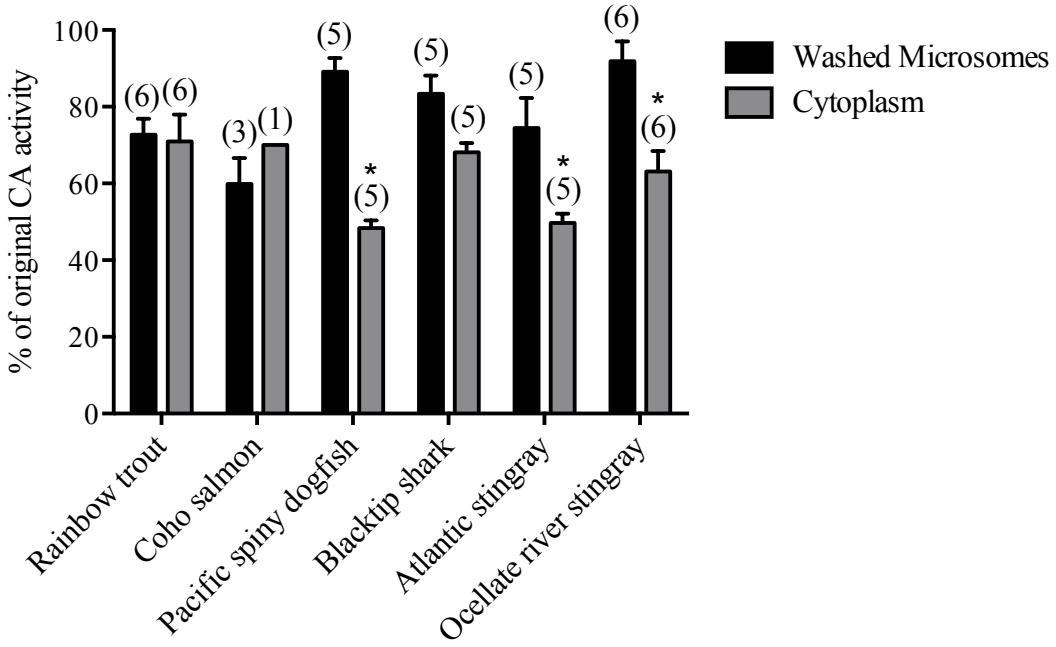
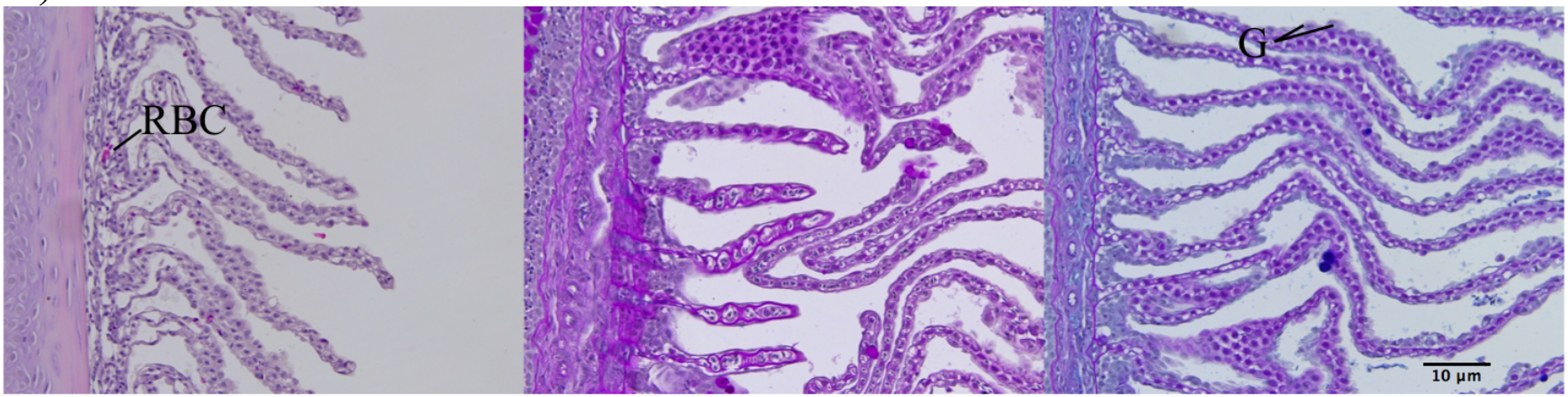
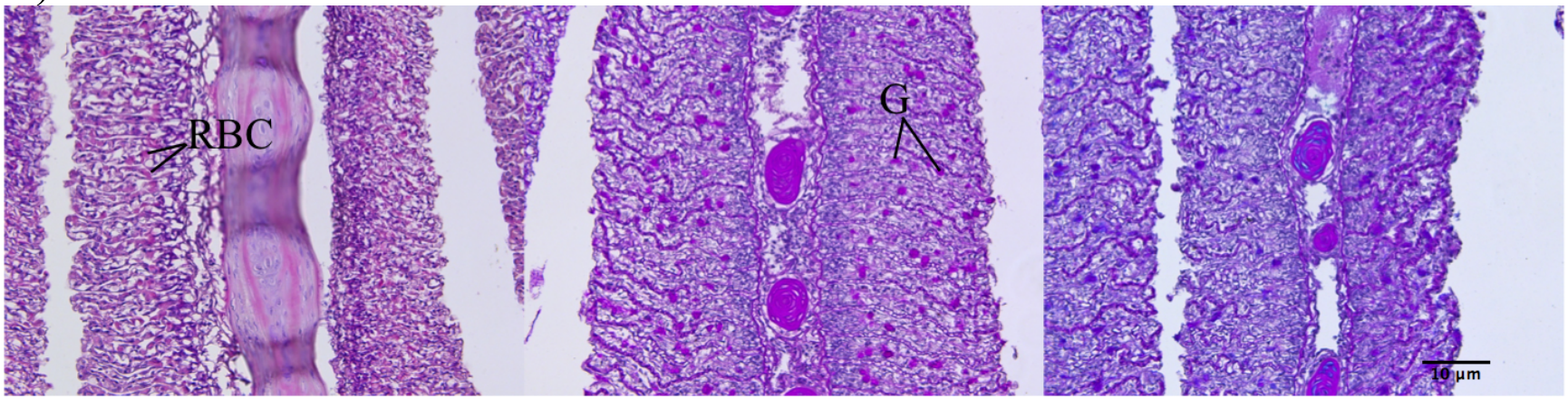


Figure 2.8. Percent (%) of original CA activity (mean ± SEM) of microsomal fraction (black bars) and cytoplasmic fraction (grey bars) after treatment with 0.005% sodium dodecyl sulphate (SDS) in two teleost and four chondrichthyan species. Higher percentage of original activity in the washed microsomal fraction than the cytoplasmic fraction indicates an SDS-resistant type IV-like CA present in gill tissue. Sample sizes are shown in parentheses and an asterisk indicates a significant difference in the effect of SDS between microsomal and cytoplasmic fractions (two sample t test, $p<0.05$)

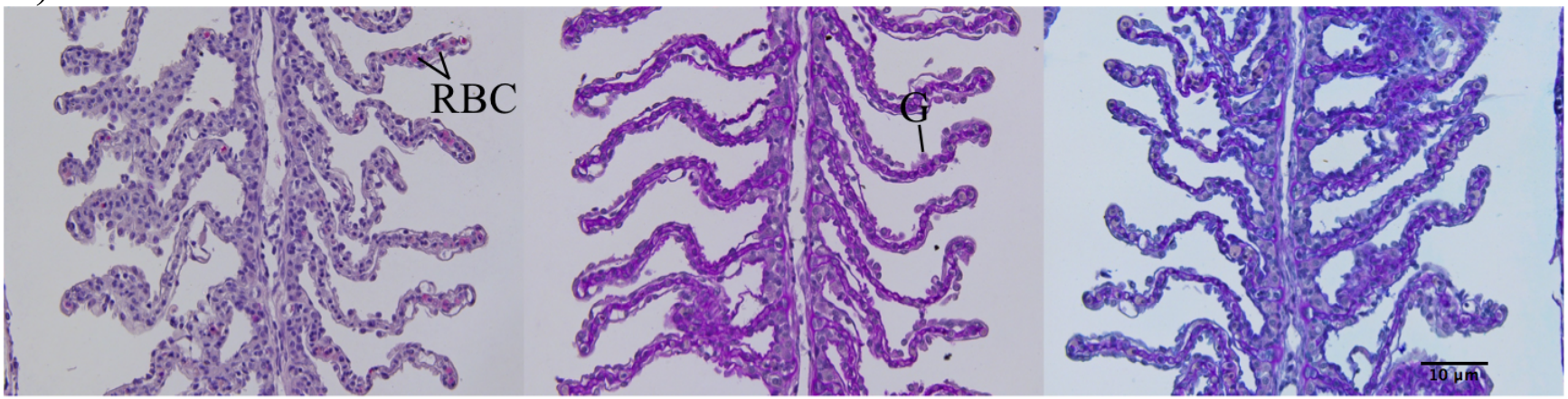
A)



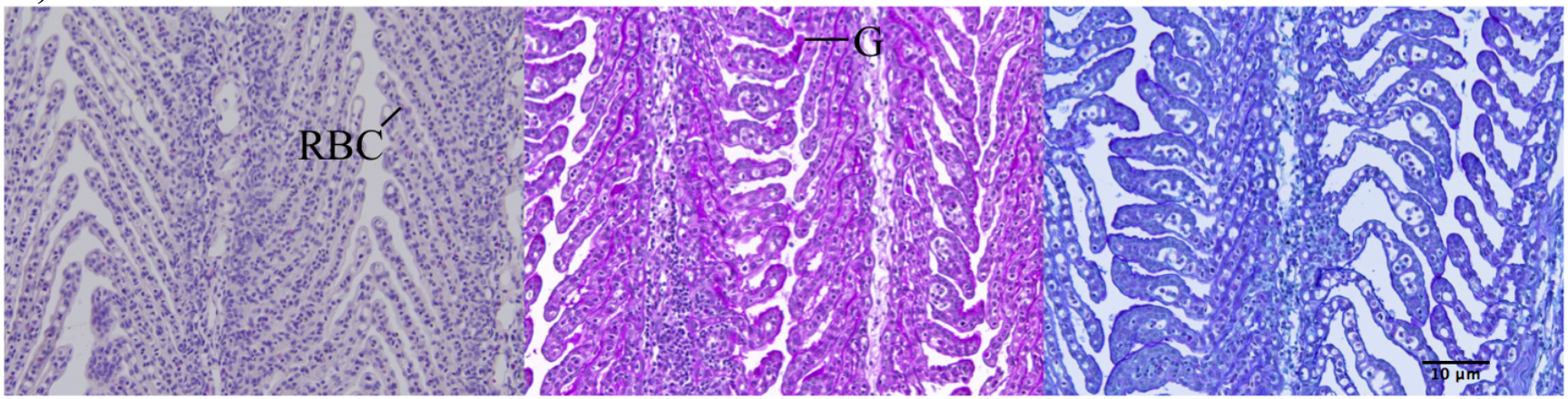
B)



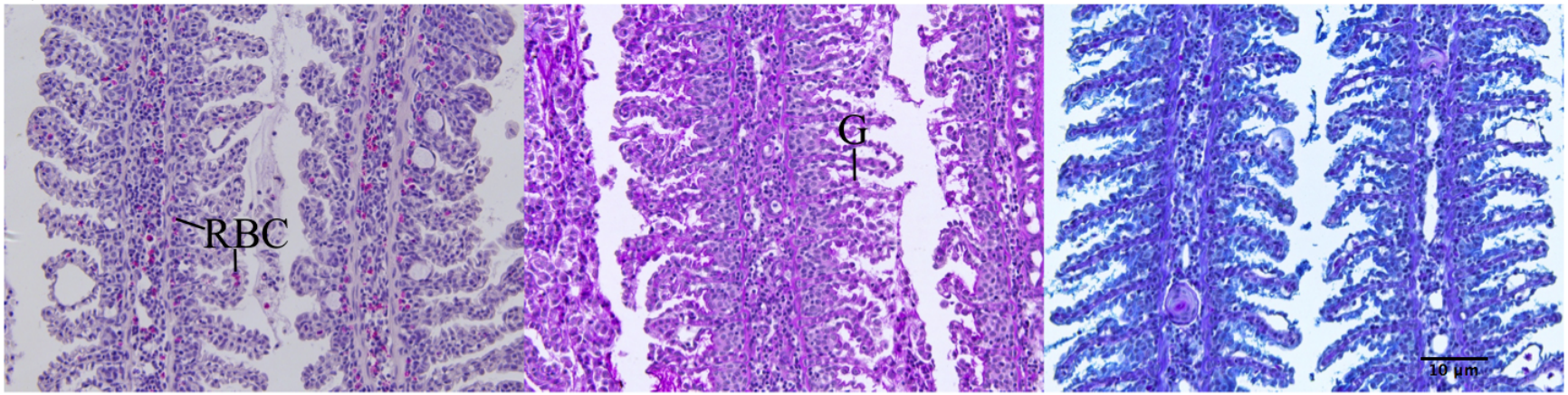
C)



D)



E)



F)

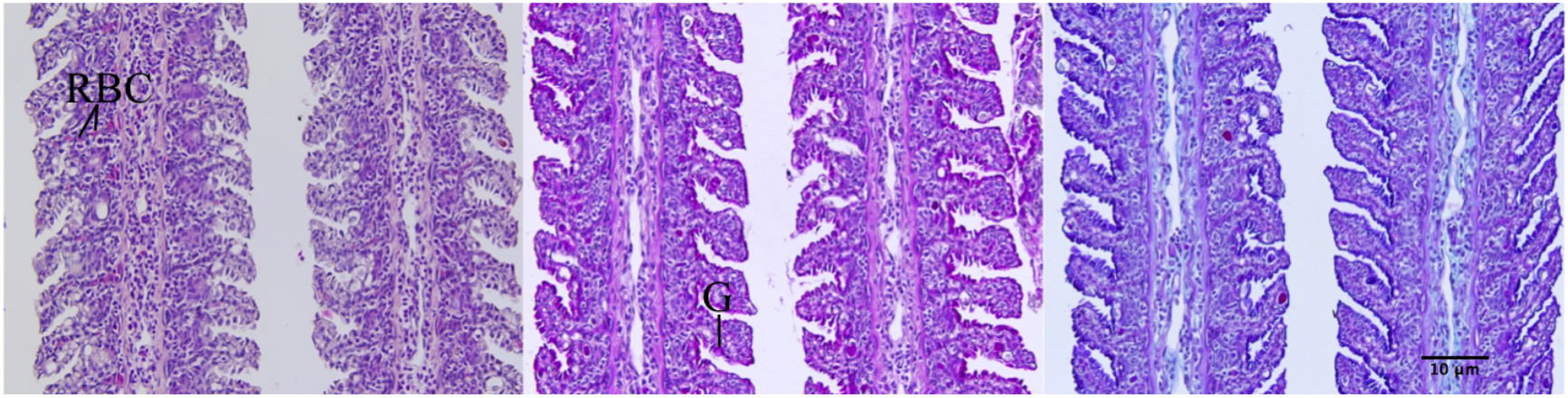


Figure 2.9. Representative histochemical images from A) rainbow trout (*Oncorhynchus mykiss*), B) coho salmon (*Oncorhynchus kisutch*), C) Pacific spiny dogfish (*Squalus suckleyi*), D) blacktip shark (*Carcharhinus limbatus*), E) Atlantic stingray (*Dasyatis sabina*), F) ocellate river stingray (*Potamotrygon motoro*). RBC = red blood cell and G = goblet cell.

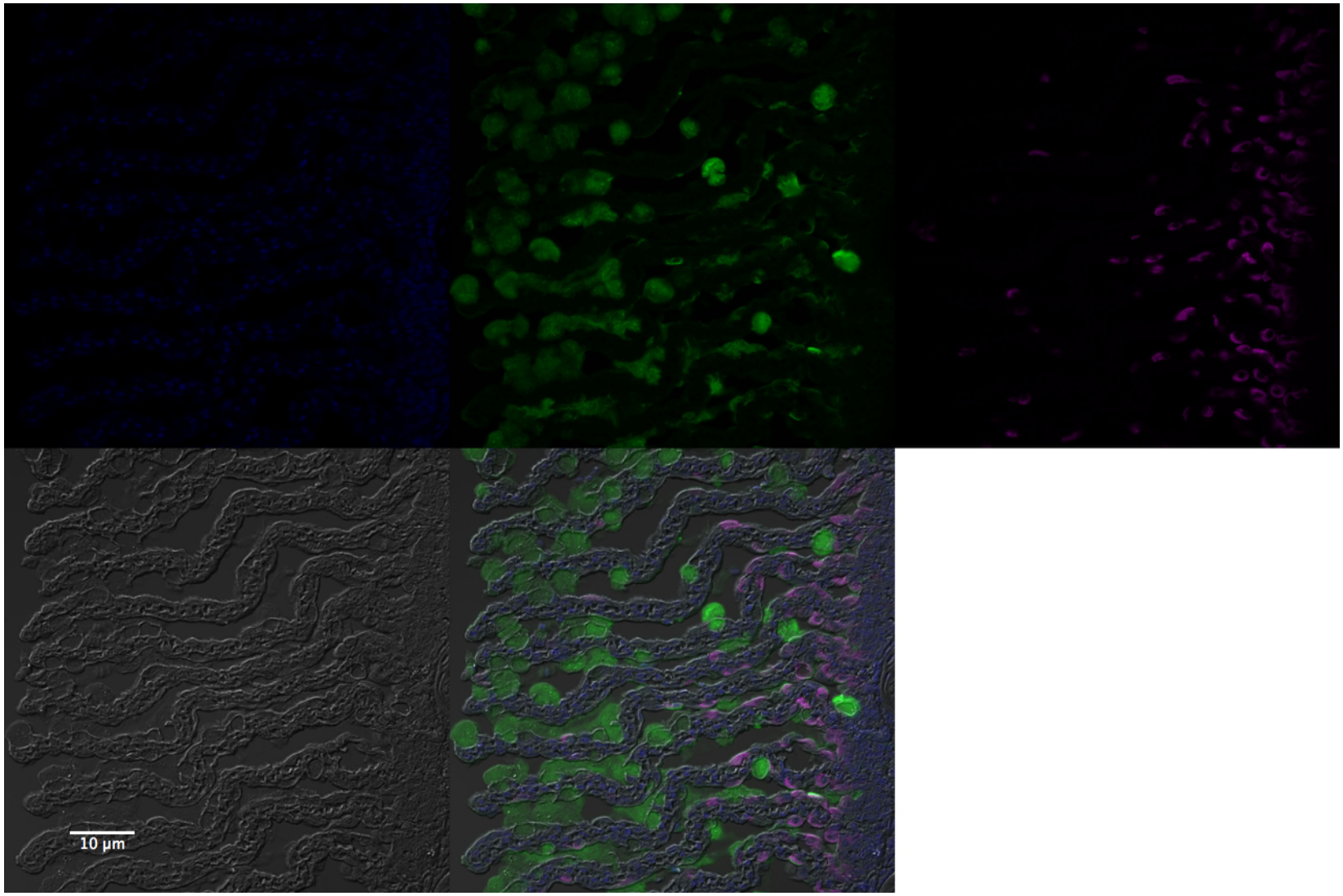


Figure 2.10. Representative immunohistochemical images of gills from rainbow trout (individual RT1; *Oncorhynchus mykiss*) showing A) 4'-6-diamidino-2-phenylindole (DAPI), B) sodium potassium ATPase (NKA), C) carbonic anhydrase (CA), D) differential interference contrast (DIC) and E) all stains together.

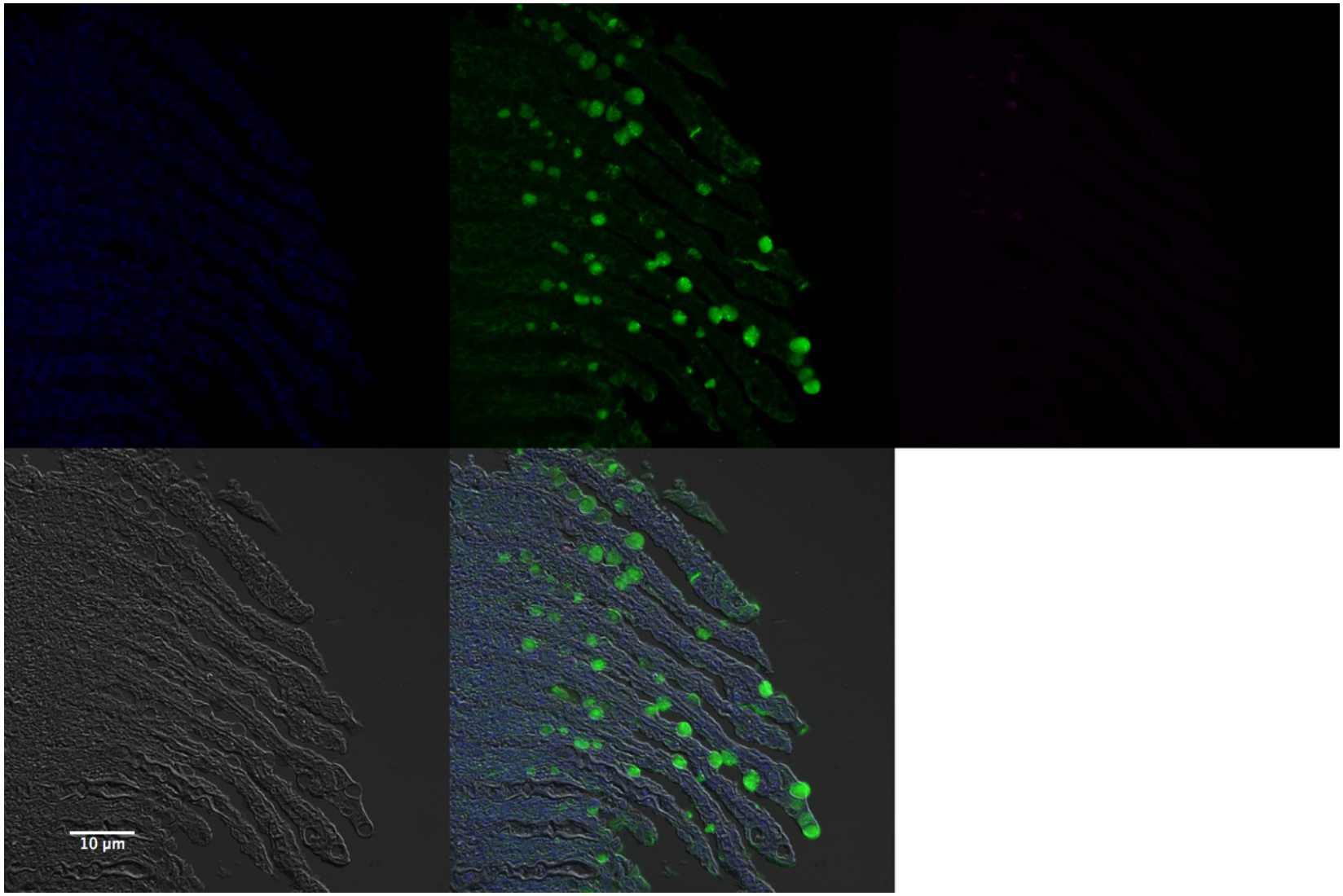


Figure 2.11. Representative immunohistochemical images of gills from coho salmon (individual C1; *Oncorhynchus kisutch*) showing A) 4'-6-diamidino-2-phenylindole (DAPI), B) sodium potassium ATPase (NKA), C) carbonic anhydrase (CA), D) differential interference contrast (DIC) and E) all stains together.

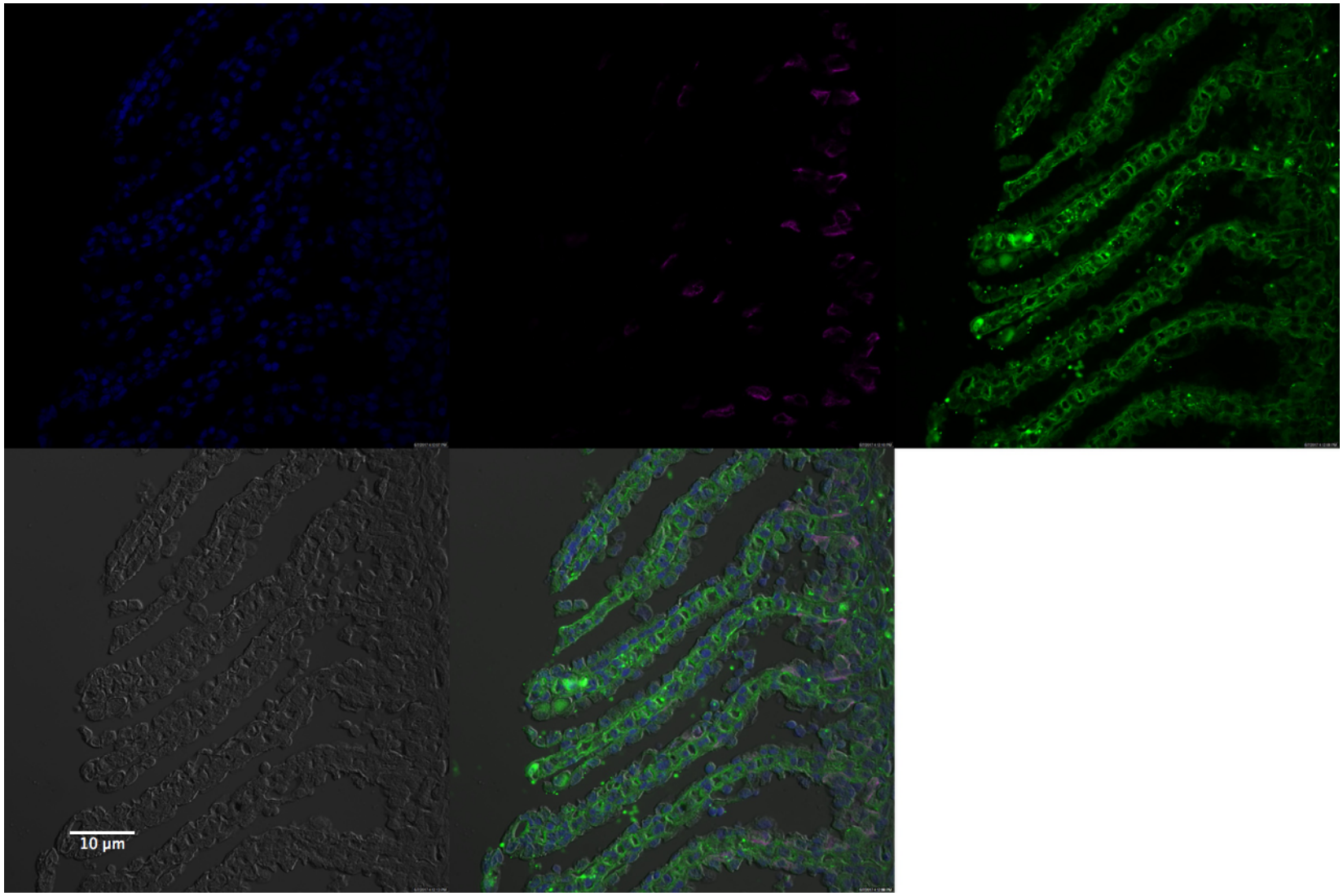


Figure 2.12. Representative immunohistochemical images of gills from Pacific spiny dogfish (individual PDF7; *Squalus suckleyi*) showing A) 4'-6-diamidino-2-phenylindole (DAPI), B) sodium potassium ATPase (NKA), C) carbonic anhydrase (CA), D) differential interference contrast (DIC) and E) all stains together.

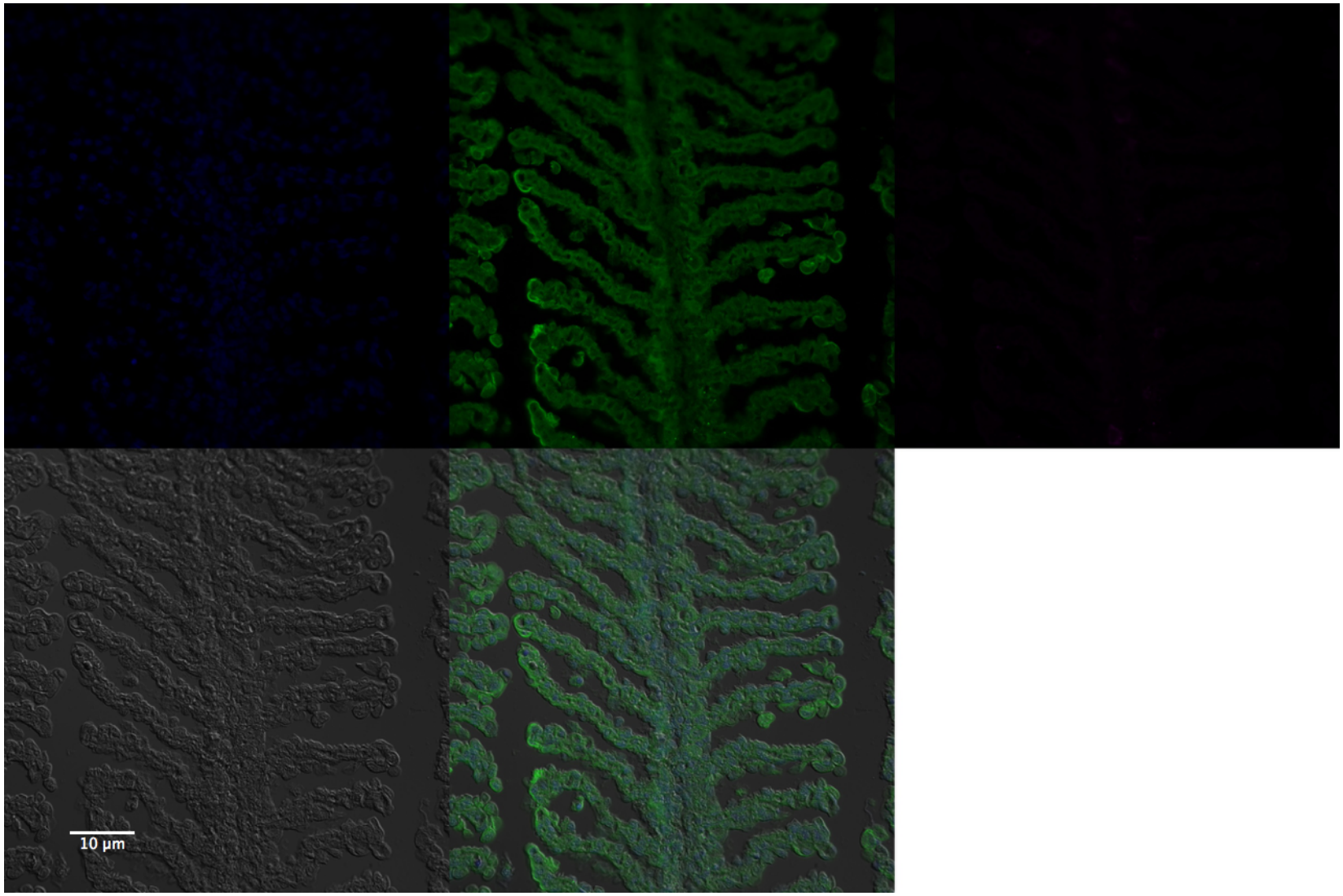


Figure 2.13. Representative immunohistochemical images of gills from blacktip shark (individual BT6; *Carcharhinus limbatus*) showing A) 4'-6-diamidino-2-phenylindole (DAPI), B) sodium potassium ATPase (NKA), C) carbonic anhydrase (CA), D) differential interference contrast (DIC) and E) all stains together.

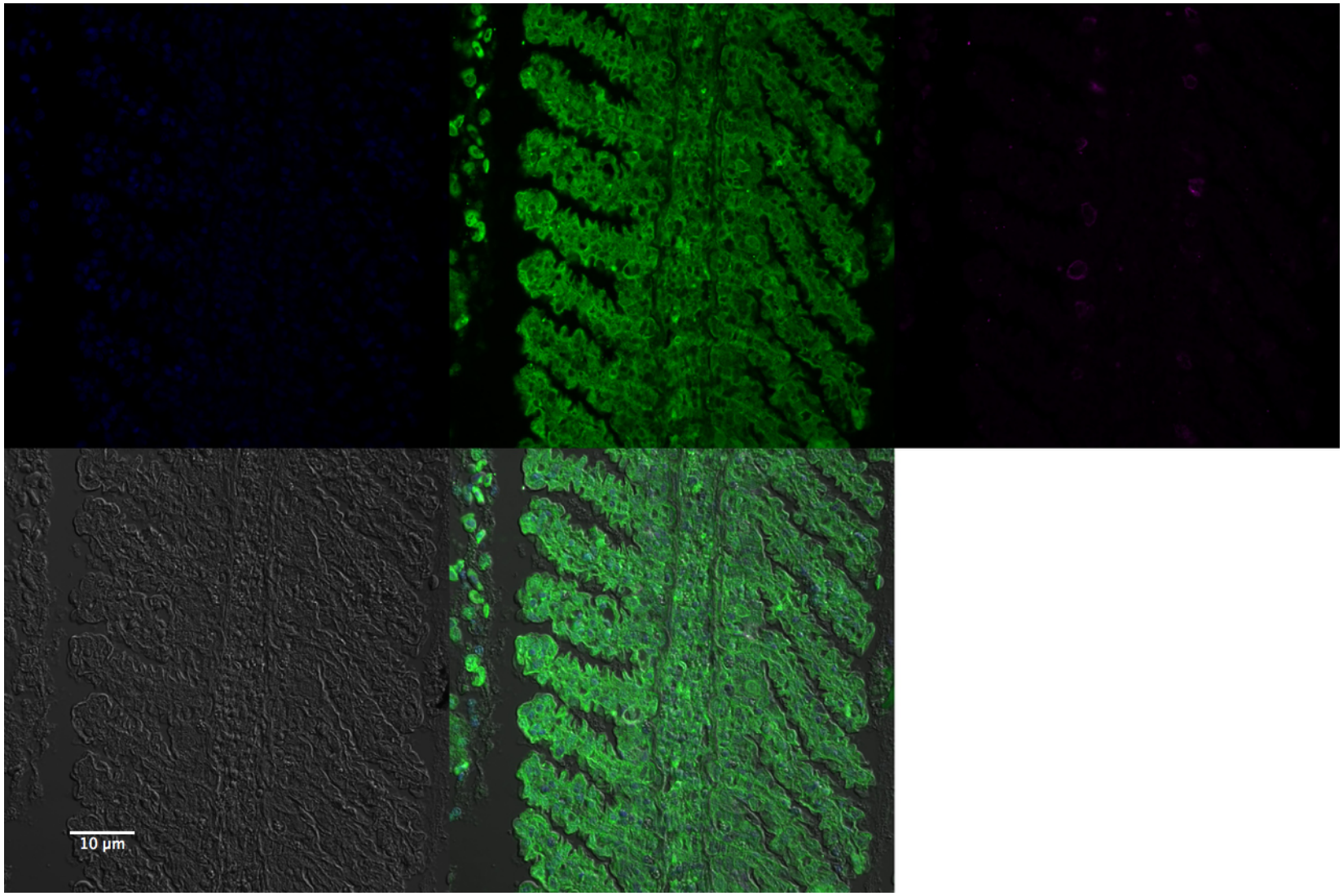


Figure 2.14. Representative immunohistochemical images of gills from Atlantic stingray (individual AS3; *Dasyatis sabina*) showing A) 4'-6-diamidino-2-phenylindole (DAPI), B) sodium potassium ATPase (NKA), C) carbonic anhydrase (CA), D) differential interference contrast (DIC) and E) all stains together.

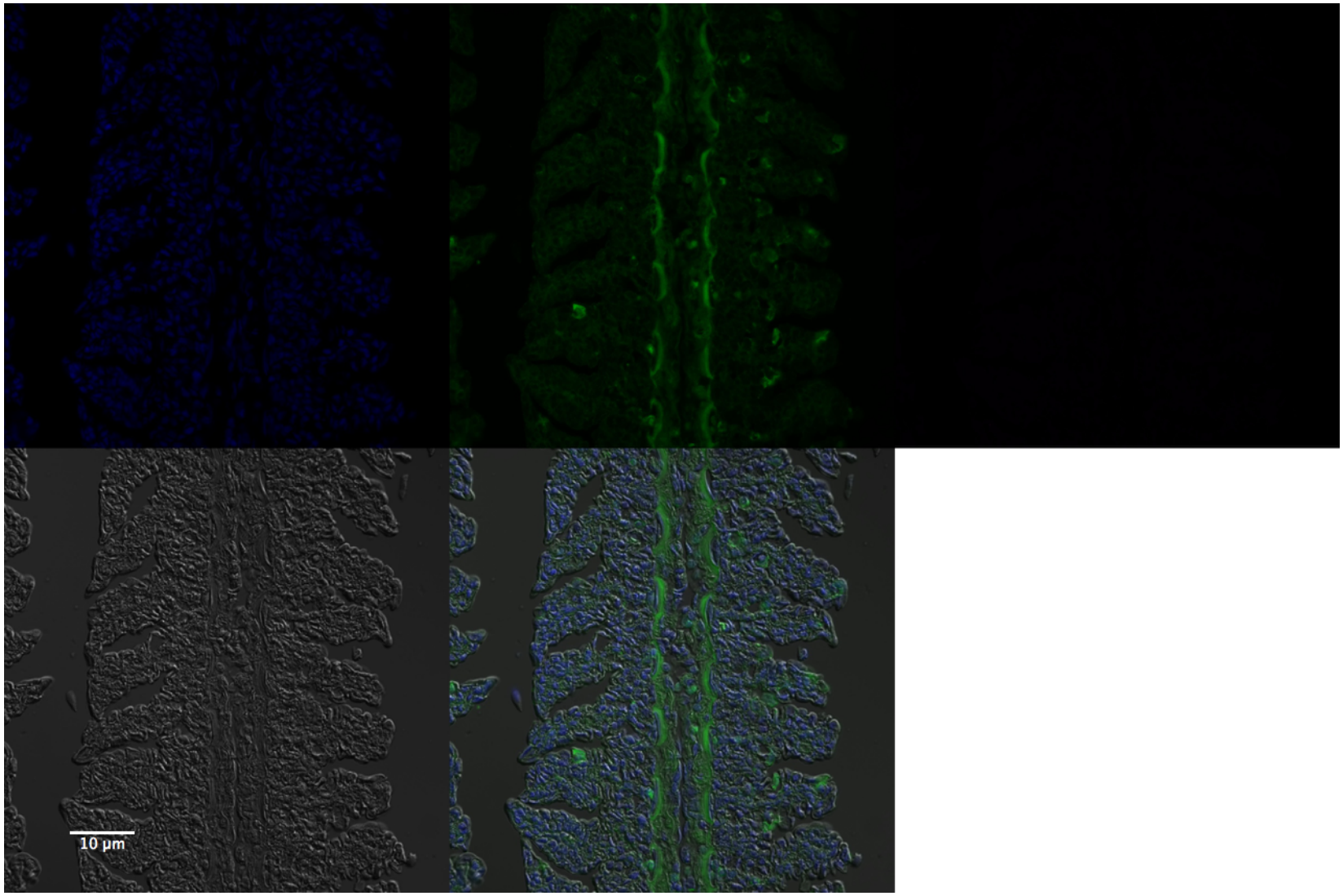


Figure 2.15. Representative immunohistochemical images of gills from ocellate river stingray (individual PM1; *Potamotrygon motoro*) showing A) 4'-6-diamidino-2-phenylindole (DAPI), B) sodium potassium ATPase (NKA), C) carbonic anhydrase (CA), D) differential interference contrast (DIC) and E) all stains together.

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Appendix

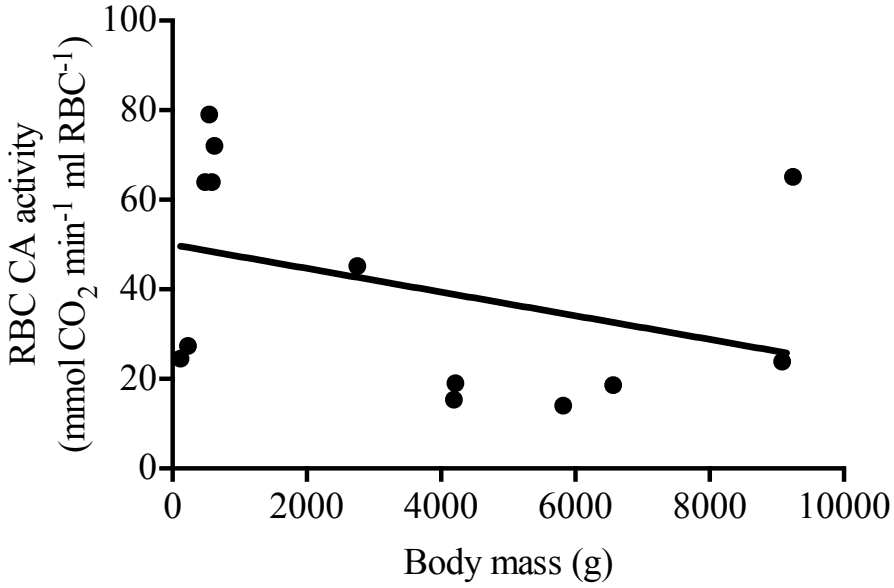

Figure A1. The relationship between RBC CA activity and body size for five chondrichthyan species. The equation of the regression line is CA activity = -0.0026*(Body mass) + 49.97, R^2=0.13, p=0.22.

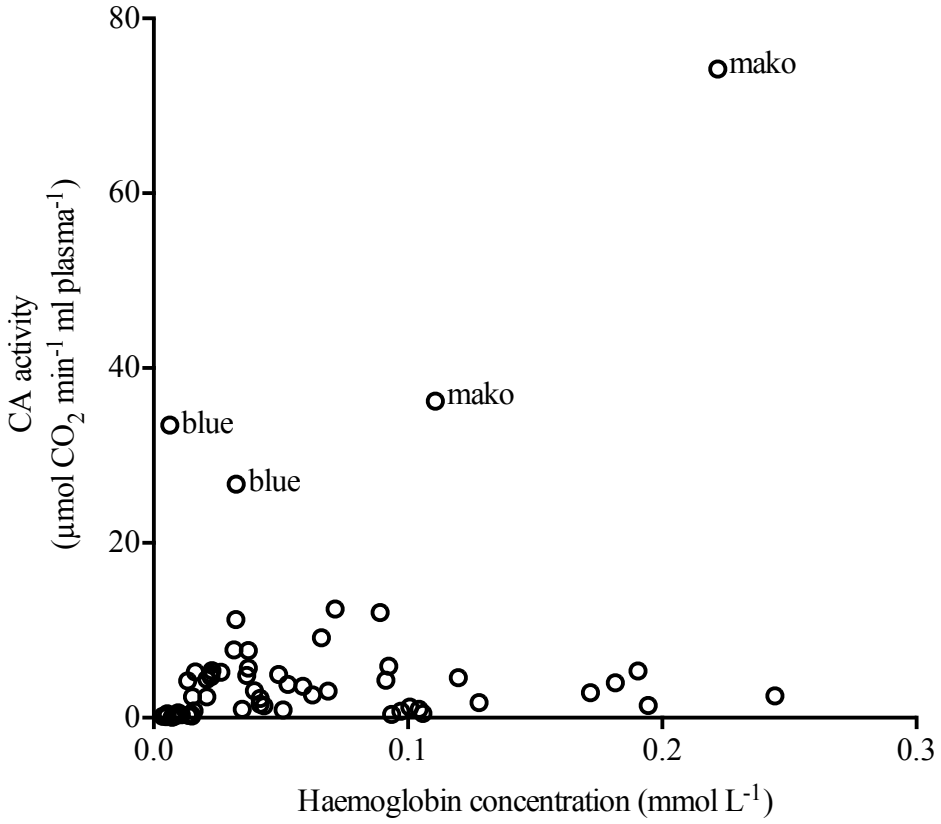

Figure A2. Relationship between plasma CA activity and hemoglobin concentration in thirteen chondrichthyan and two teleost species. Labels blue=blue shark (*Prionace glauca*) and mako=shortfin mako shark (*Isurus oxyrhinchus*).

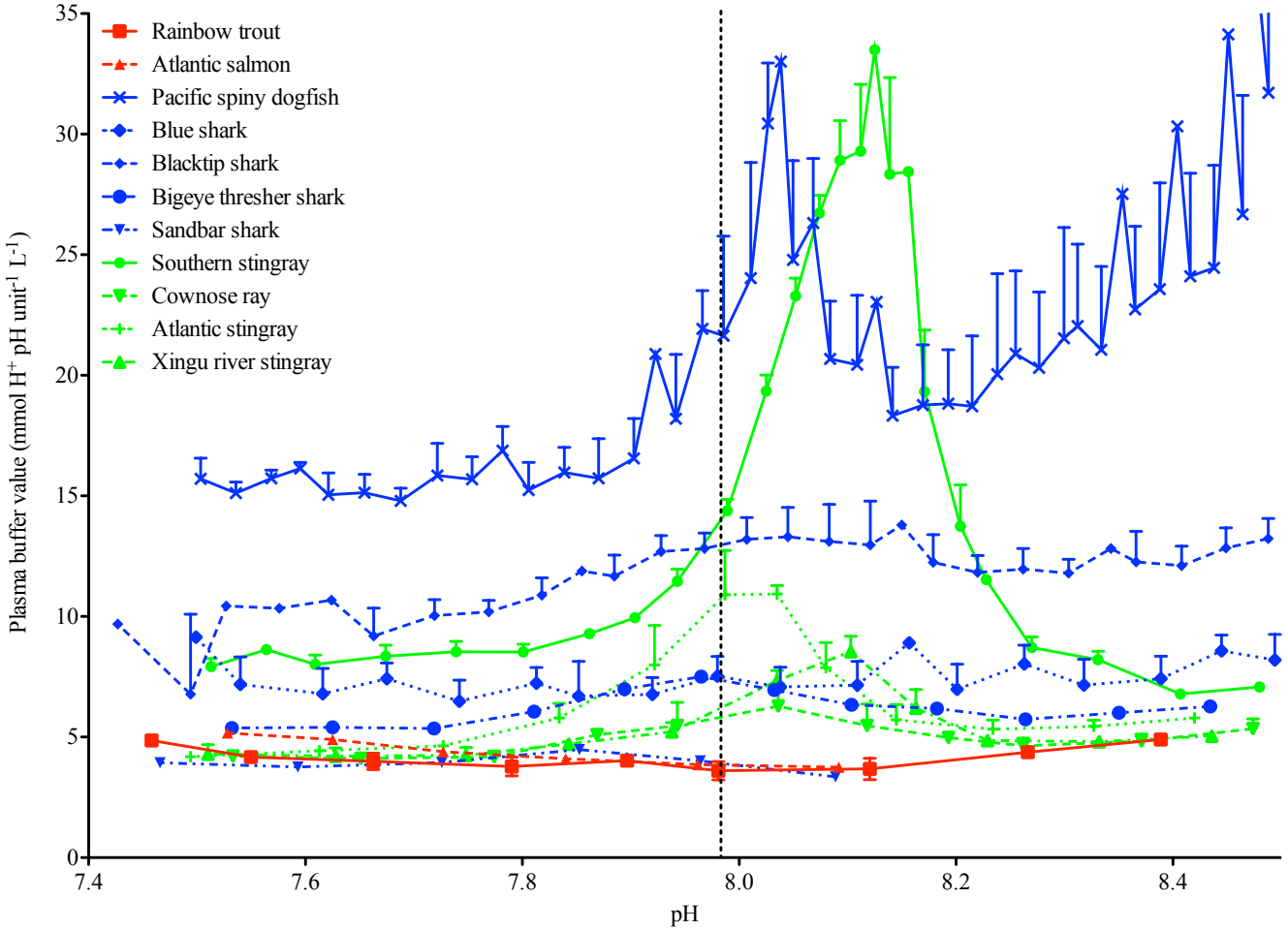

Figure A3. Instantaneous plasma buffer values from pH 7.5-8.5 for eight chondrichthyan and two teleost species. The vertical line at pH 8 indicates the value taken for species comparisons in Figure 2.5. Red traces are teleosts, blue are sharks and green are rays.