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Whole-animal to molecular studies of solute transport in fishes

by

Samuel Clark Guffey

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Abstract

I have examined solute transport in two fishes at the whole-animal, tissue, and molecular levels. In the Pacific hagfish, I measured the kinetics of phosphate absorption in isolated gut, skin, and gill preparations, and implicated an NaPi-II transporter based on RT-PCR results and the kinetics of phosphate uptake. In a second series of experiments, subjecting Pacific dogfish to reduced salinity revealed that plasma sodium and chloride homeostasis are maintained. Therefore these ions are not a limiting factor in reduced salinity; however, urea efflux significantly increased over the 48 hour exposure. Finally, I investigated the molecular physiology of sodium homeostasis by cloning Na^+/H^+ Exchanger [NHE] isoforms 2 and 3 from the dogfish gill and expressing them in NHE-deficient AP-1 cell lines. The sensitivity to inhibitors was found to be different from mammalian NHEs, revealing appropriate concentrations to use for elasmobranchs and other fishes, and allowing future mechanistic studies to be conducted.

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CDS (coding sequence)

Cl^- (chloride)

FW (freshwater)

H^+ (proton or hydrogen ion)

HCO_3^- (bicarbonate ion)

mmol (millimoles)

mOsm (milliosmoles)

Na^+ (sodium)

NH_3 (ammonia)

NH_4^+ (ammonium)

NHE (sodium/proton exchanger)

NKA (sodium/potassium ATPase)

P (partial pressure)

PCR (polymerase chain reaction)

pH_i (intracellular pH)

P_i (inorganic phosphate)

ppt (per mille or parts per thousand)

‰ (per mille or parts per thousand)

RACE (rapid amplification of cDNA ends)

SEM (standard error of the mean)

SW (seawater)

VHA (V-type H^+ ATPase)

[x] (concentration of substance x)

Chapter I

General introduction

Homeostasis and some challenges of an aquatic existence

All known organisms tend to maintain a relatively stable composition of the internal environment within the body or within the cell (Bernard 1878). This idea was termed homeostasis by Cannon (1929), who further refined the term to denote the tendency of a system to act to regulate its internal environment in order to maintain stability despite changes in the external environment.

The composition of this internal environment is different from that of the external medium, whether the latter is an extracellular fluid within the organism or the external fluid environment bathing the organism. The extracellular fluids of fishes are generally very similar to the blood plasma, which may or may not differ greatly in composition from that of the external fluid medium, depending on the type of fish. Table 1.1 describes the typical composition of seawater (SW), freshwater (FW), and intracellular fluid and blood plasma from several species of fishes.

In the case of freshwater and marine fishes, the composition of the external environment may influence that of the internal environment through two main routes: (1) ingestion of the external medium with or without food and (2) constant impingement of the medium upon every exposed body surface (Burger 1967). While ingestion of food and of the external medium are more or less controlled by the animal, constant contact with the external medium creates an unavoidable flux of materials between the fish and the medium. With its high surface area, thin membranes, and high degree of perfusion present, the gill is the main site for this flux (Bentley et al. 1976; Krogh 1939). Dissolved materials tend

to move down their concentration gradient or electrochemical gradient in the case of charged particles. Thus, an elasmobranch such as *Squalus acanthias* exposed to SW will tend to receive an influx of all of the major ions in SW. High concentrations of urea and trimethylamine oxide (TMAO) present in the plasma result in a plasma osmolality roughly matching that of SW, thus minimizing osmotic water movement. However, the absence of urea and TMAO in SW leads to a large outward concentration gradient and thus considerable efflux of these compounds from the animal. In the case of exposure to FW or very dilute SW, the animal would then suffer efflux of all major solutes along with an osmotic influx of water. In order to maintain a constant internal environment, the inward and outward fluxes of each of these materials must be balanced in the long term. While urea (and possibly TMAO) can be produced by the animal, all other components must be managed through a balance of ingestion, passive flux, and excretion.

With respect to phosphate, which is simultaneously a major solute, a pH buffer, and a nutrient, further challenges to homeostasis arise. This component is present in micromolar concentrations in SW but necessary in millimolar concentrations within the body (Currie and Edwards 2010). Furthermore, as a nutrient, it is also consumed by cells as it is incorporated into nucleic acids and mineralized tissues. This necessitates a balanced intake of phosphate even in the presence of minimal phosphate efflux. The intake of phosphate is usually assumed to be almost entirely through ingestion of food, and considerable effort has been

expended in attempts to enhance the efficiency of phosphate intake in aquaculture (Hernandez et al. 2005; Sarker et al. 2011; Wang, et al. 2012).

Study animals

I have investigated some of these challenges to solute homeostasis in two very different fishes, the Northeastern Pacific hagfish *Eptatretus stoutii*, and the North Pacific spiny dogfish *Squalus suckleyi*.

The Northeastern Pacific hagfish, Eptatretus stoutii

The hagfish *E. stoutii* is exemplary of a group of jawless fishes having elongate, scaleless bodies, six to eight pairs of internal gill pouches, a cartilaginous cranium, and no spine, bones or paired fins. This strictly marine species lives on the seafloor at depths of 10-700 m in the cold temperate waters of the northeastern Pacific Ocean from Alaska to Baja California (Fernholm 1998).

The hagfishes, as a group, appear to have diverged evolutionarily from the remainder of the vertebrate lineage around 500 million years ago and have remained morphologically very similar for at least the last 305 million years (Janvier 2007) (Figure 1.1). As the hagfishes lack many of the characteristics of other vertebrates, they have been called the sister group to the other vertebrates. Together with the related lampreys, which share several morphological characteristics with the hagfishes, all extant jawless fishes have been united under the clade Cyclostomata (Janvier 2007). Morphological and developmental evidence strongly suggest that the hagfishes are vertebrates; *e.g,.* Ota et al. (2007) reported similar development in the neural crest in the embryos of hagfishes and gnathostomes, Ota et al. (2011) reported the presence of cartilaginous vertebral

elements in hagfishes and a report by the same authors in 2013 provided evidence that these hagfish vertebral elements arise from slcerotomes just as they do in gnathostomes. However, the monophyly of Cyclostomata remains the subject of debate. For a current and thorough review of this debate, see Miyashita (2012). Most molecular analyses support cyclostome monophyly. Heimberg et al. (2010) supported monophyly based on the shared presence of four microRNA families and 15 paralogs between hagfishes and lampreys that are not found in the gnathostomes. Comparison of 18S rRNA sequences by Stock and Whitt (1992) also supported monophyly. Additional analysis of 28S rRNA sequences by Mallat and Sullivan (1998) supports the same conclusion. Yu et al. (2008) analyzed 34 nuclear gene families and found support for monophyly, but their analysis of 14 mitochondrial gene families more strongly supported cyclostome paraphyly.

However, many character-based analyses support different conclusions. In Miyashita's (2012) review, it is claimed that the support provided by recent molecular studies is overestimated and the result of a new morphological analysis provides weak but unambiguous support for cyclostome paraphyly. A study by Oisi et al. (2013) refers to the developmental migration of myotomes, velum and tongue apparatus as a cyclostome synapomorphy, but the overall developmental plan of the cyclostome head may in fact be a plesiomorphy or ancestral trait of all vertebrates. Other morphological studies tend to separate hagfishes and lampreys. Lovtrup (1977) lists several morphological characteristics that unite lampreys and gnathostomes as separate from hagfishes. Several morphological and

developmental studies by Hardisty (1982), Maisey (1986), and Janvier (1996a, b) also support paraphyly of Cyclostomata.

In the end, if Cyclostomata is truly monophyletic, this would imply that a large number of hagfish characteristics are either unique to hagfishes or reversions to a more primitive state, or alternatively, that lampreys and gnathostomes share several similar but independently evolved traits. These include, among others, absence of vagal innervation of the hagfish heart, implying either loss in the hagfish or convergent evolution in the other vertebrates; osmoconformity along with apparent lack of sodium and chloride homeostasis in the hagfish, while lampreys and gnathostomes both exhibit osmoregulatory strategies that, as far as has been investigated, appear to operate through fundamentally similar mechanisms; and a complete lack of freshwater or anadromous forms throughout modern and fossil hagfishes, in contrast to almost every other living fish group and many fossil forms as well (Janvier 2007). While the absence of the eye lens, lateral line neuromasts, and solidified vertebrae have been more or less accepted as either reversions or hagfish apomorphies (Stockard 1906, Wicht and Northcutt 1995, Ota et al. 2011), the previously mentioned traits of uncertain evolutionary history are remarkable characteristics of a modern organism that is considerably less complex than other living vertebrates.

For those who refute cyclostome monophyly and consider the hagfishes to be the earliest-diverging extant fishes, the hagfishes are useful to researchers seeking to examine characteristics of the ancestral vertebrate. However, it is also possible that the hagfishes acquired derived characteristics that would be

inappropriate to assign to the ancestral vertebrate. If Cyclostomata is truly monophyletic, the characteristics of the hagfish may inform our understanding of, though they may not be identical to, the characteristics of the ancestral vertebrate.

Regardless of its phylogeny, the extant hagfish exhibits several peculiarities that make it an interesting study species from a physiological point of view. First, it is the only known fish to exhibit both osmoconformity and ionoconformity with respect to the major ions of SW (Currie and Edwards 2010; Wright 2007) (Table 1.1). The only ions for which the hagfish maintains lower concentrations in the plasma than in SW are calcium, magnesium, and sulfate. Thus, the mass transfer of sodium, chloride, potassium, and water between the animal and the environment is minimized. As a result, the hagfish is able to exhibit a high permeability to water and partial permeability to sodium and chloride (McFarland and Munz 1965).

Another peculiarity lies in the hagfish's mode of feeding. Although *E. stoutii* is found to feed often on polychaetes, and another species has been filmed capturing and eating small fishes (Zintzen et al. 2011), the hagfishes are also known as major consumers of seafloor carrion (Bardack 1998; Martini 1998). In the case of large dead fishes, sharks, and whales, the hagfish is known to enter through the mouth, anus, or a wound and consume the carrion from within (Hart 1973; Linnaeus 1758).

This unusual feeding mode, coupled with the partial permeability of the animal, recently led several researchers to investigate the possibility of nutrient transport across the body surfaces. It was demonstrated that *E. stoutii* can absorb

the amino acids glycine and alanine through the skin and gill when these nutrients are available in the water, as they are likely to be within a decomposing animal (Glover et al. 2011). This was the first demonstration of organic nutrient transport across the skin and gill of a chordate species, and it paves the way for investigations into the transport of other materials in this unusual animal.

The North Pacific spiny dogfish, Squalus suckleyi

The dogfish *Squalus suckleyi* is known for being commonly studied as an example of elasmobranch anatomy. The closely related species *Squalus acanthias* has often had its name applied as a senior synonym to *S. suckleyi*, but Ebert et al. (2010) re-established the latter as a distinct species. In fact, genetic evidence supports a North Pacific origin for modern dogfish, with colonization of other basins likely occurring at least seven million years ago (Verissimo et al. 2010). These sharks belong to the type genus of one of the two major clades of modern sharks, Squalomorphii. The chondrichthyan lineage, which includes elasmobranchs and holocephalans (which will not be discussed further), diverged from the rest of the vertebrate lineage approximately 400 million years ago (Figure 1.1) (Benton 2005). The Squalomorphii diverged from the other major clade of modern sharks, Galeomorphii, approximately 150-250 million years ago (Benton 2005; Velez-Zuazo and Agnarsson 2011).

As a representative marine shark, *S. suckleyi* regulates the composition of its body fluids as shown in Table 1.1, and therefore faces the above mentioned challenges with respect to solute homeostasis in SW. The basic mechanisms by which SW elasmobranchs maintain solute homeostasis have been described

primarily from experiments on *S. suckleyi* and *S. acanthias* (Burger 1967). Influxes of sodium and chloride are dealt with by a uniquely chondrichthyan organ, the rectal gland, which secretes a concentrated solution of sodium chloride to precisely balance sodium and chloride influx (Burger and Hess 1960). This, in combination with the kidney, also accomplishes the task of balancing the minor water influx due to osmosis and drinking (Anderson et al. 2007; Burger 1965; Forster 1967).

The potential for urea efflux is considerable because of the large concentration gradient between animal and water. Urea efflux is minimized by a low permeability of the gills to urea (Boylan 1967; Wood et al. 1995) and by resorption of filtered urea within the kidney tubule (Forster et al. 1972; Goldstein et al. 1968; Hays et al. 1977; Perlman and Goldstein 1988; Yamaguchi et al. 2009). To balance losses, elasmobranchs produce urea in the liver and muscle (Anderson et al. 2005; Goldstein 1967; Hazon et al. 2003; Steele et al. 2005) and can absorb urea across the intestine after consuming urea-rich food (Liew et al. 2013).

In the wild, dogfish sharks and other elasmobranchs may occasionally encounter brackish or diluted SW when approaching or entering estuaries. In this diluted environment, the concentration gradients for sodium and chloride entry are reduced or even reversed (see Table 1.1, brackish water and FW). The osmotic gradient for water entry is increased, and high concentrations of plasma urea exacerbate this problem. Therefore, physiological adjustments must be made if the animal is to remain in such an environment for more than a short time. Despite

these challenges, some elasmobranchs have been found far up estuaries and in completely fresh water (Ballantyne and Fraser 2012; Piermarini and Evans 1998; Smith 1931a; Thorson 1967). As early as 1931, Homer Smith was able to make general conclusions about the homeostatic strategies exhibited by SW and FW elasmobranchs based on his observations and experiments on many elasmobranch species around the world (Smith 1931a,b). Elasmobranchs in FW exhibit around a 30% reduction in sodium chloride concentration and a 50% reduction in urea concentration in the plasma compared to SW elasmobranchs, resulting in a reduced osmotic gradient for water influx (Smith 1931a,b). It was later discovered that several species, including some of those studied by Smith, are able to migrate between SW and FW and are therefore fully euryhaline (Ballantyne and Fraser 2012; Piermarini and Evans 1998; Thorson 1958; Thorson 1962). Many other species have been studied as examples of either marginally euryhaline elasmobranchs or as stenohaline marine elasmobranchs in order to observe the effects of acute exposure to slightly diluted SW (Bedford 1983b; Burger 1965; Burger 1967; Burger and Tosteson 1966; Chan and Wong 1977; 2004; Forster et al. 1972; Morgan et al. 1997). Although some of the physiological responses are at least qualitatively similar in many elasmobranchs, general descriptions of the effects of dilution on elasmobranchs rely on several assumptions including but not limited to cross-species inference. Reviews still struggle to explain why only a few elasmobranchs are euryhaline and the rest are more or less stenohaline. While it has been proposed that the barrier to euryhalinity is a quantitative problem of balancing solute influx versus efflux (Choe et al. 2007; Evans 2008), the relevant

physiological factor limiting brackish or FW incursions has not been established. Adding to this puzzle, even stenohaline elasmobranchs possess the transport protein that is supposed to be responsible for sodium uptake in FW, a Na^+/H^+ exchanger [NHE] (Choe et al 2007). Analyzing the functional properties of this protein and other solute transport proteins may contribute to the resolution of this problem.

Incorporating molecular physiology

In recent decades, fish physiology, and comparative physiology in general, has lagged behind biomedical physiology in incorporating the techniques of modern molecular biology. Following the discovery of the first ion-transporting protein, the Na^+/K^+-ATPase (Skou 1965), research at the level of individual protein molecules has been increasingly applied in attempts to reduce the patterns of solute transport observed in whole animals, organs, and tissues to a function of transport mechanisms operating at the cellular level. Using principles of molecular biology that were established in the 1970s and 1980s, transport proteins can now be studied in isolation in order to determine their structural and functional properties (Hediger et al. 1987; Kopito and Lodish 1985; Orlowski 1993).

In the technique of molecular cloning, protein-coding RNA isolated from an organism is first copied into more stable cDNA. By identifying and isolating the cDNA sequence that encodes a protein of interest, the amino acid sequence of the protein can be inferred. The primary structure of various proteins can then be compared and contrasted in an attempt to deduce the importance of various

segments (Kopito and Lodish1985). Knowledge of the primary structure can also be used to generate antibodies against a specific protein for application in many common immunological-based techniques (western blot, immunohistochemistry, radioimmunoassay, ELISA, *etc.*).

Functional analysis of the cloned protein can be accomplished through expression cloning. In this technique, the isolated protein-coding sequence is introduced into cells such as *Xenopus* oocytes or a cultured cell line (Hediger et al. 1987; Orlowski 1993). By expressing large amounts of the cloned protein in these cells, the functional properties of the protein can be determined. In the case of transport proteins, the flux of substances in or out of the cell as a result of the introduced transport protein can be measured (Franchi et al. 1986; Orlowski 1993). Thus, the overall transport kinetics and apparent affinity of the protein for its substrate(s) can be determined. This also provides an opportunity to test the effects of various compounds on transport activity in order to find drugs that inhibit particular transport proteins.

Na^+/H^+ exchangers

One type of transport protein that is a focus of this thesis is the Na^+/H^+ exchanger, or NHE (HUGO gene nomenclature: SLC9A subfamily). As suggested by the name, it catalyzes the electroneutral exchange of one extracellular sodium ion for one intracellular proton. The known functions of NHE include the regulation of cell volume and intracellular pH (Boron and Boulpaep 1983; Grinstein et al. 1983), and transepithelial sodium transport (Knickelbein et al. 1983), and it has also been implicated in systemic pH balance

and sodium uptake in fishes (Dymowska et al. 2012; Evans 1982; Krogh 1939). In mammals, there are at least nine functional NHE genes, named NHE1-9 (SLC9A1-9) (Donowitz et al. 2013). In chondrichthyan fishes, eight NHE isoforms (NHE1, 2, 3, 5, 6, 7, 8, and 9) have been identified in the draft genome sequence of the holocephalans elephant shark, *Callorhinchus milii* (Venkatesh et al. 2007), whereas mammalian NHE2 and NHE4 apparently diverged after the separation of chondrichthyans from the ancestor of tetrapods. All NHEs are integral membrane proteins and can be classified according to their location within the cell. NHE1-5 reside in the cell membrane, while NHE6-9 are located primarily in intracellular organelles (Brett et al. 2005). The cell membrane NHEs are further divided into a recycling clade, including NHE3 and 5, and a resident clade, including NHE1, 2, and 4 (*ibid.*) The resident NHEs for the most part remain in the cell membrane after insertion, while the recycling NHEs are frequently retrieved from the cell membrane into recycling endosomes until signals once again initiate their insertion (Alexander and Grinstein 2009).

The discovery of NHEs in fishes was preceded by repeated observations of macroscopic Na^+/H^+ exchange. Early work by Krogh (1939) demonstrated that sodium uptake in fishes was associated with equal and opposite acid (H^+ or NH_4^+) efflux and later studies determined that several fishes are unable to excrete acid in the absence of sodium (Claiborne et al. 1994; Evans 1982). The first piscine NHE to be identified, NHE-β (Borgese et al. 1992), was isolated from the trout erythrocyte, but this could not account for the observations of macroscopic Na^+/H^+ exchange because it was not found in any cells that abut the external

medium. A few years later, apparently medium-accessible isoforms of NHEs were cloned and identified in ionoregulatory cells within the gill epithelium of several species (Claiborne et al. 1999; Edwards et al. 1999). This was soon followed by the immunological identification (Edwards et al. 2002) and later cloning (Choe et al. 2005) of similar NHEs in elasmobranchs. All of the NHEs cloned from fish gills have been homologues of either NHE2 or NHE3, and attempts to localize other NHE isoforms to gill cells have returned negative results (Yan et al. 2007). In elasmobranchs, NHE2 and NHE3 have been implicated in various homeostatic functions including systemic pH balance (Claiborne et al. 2008; Tresguerres et al. 2005) and possibly sodium uptake in brackish or FW (Choe et al. 2007; Choe et al. 2005; Reilly et al. 2011; Wood et al. 2002). Therefore, NHE2 and NHE3 are the isoforms investigated in the last part of this thesis.

As mentioned above, NHE2 and NHE3 have been implicated in various homeostatic functions in fishes, but it has thus far been impossible to prove their role. Previous studies rely on, at worst, analogy to other epithelia in order to explain NHE localization (Choe et al. 2007; Claiborne et al. 2008; Edwards et al. 1999; Edwards et al. 2002). Some report correlative observations of either NHE mRNA or protein abundance in response to either acidosis or brackish water challenge (Choe et al. 2005; Claiborne et al. 1999; Ivanis et al. 2008; Reilly et al. 2011; Tresguerres et al. 2005; Yan et al. 2007). Still others try to analyze the role of NHEs by application of inhibitory drugs (Brix and Grosell 2012; Kirschner et al. 1973; Parks et al. 2009; Preest et al. 2005; Wood et al. 2002). In the absence of a genetic knockout or knockdown, which may never become available for animals

as large as elasmobranchs, the application of inhibitors may seem to be the most promising tool for dissecting the contributions of NHEs. However, the efficacy and specificity of common inhibitory drugs on fishes are unknown.

The latter quandary can be definitively settled through the techniques of molecular physiology. By isolating NHEs from fishes and expressing them in active form, the efficacy and specificity of drugs can be precisely determined. Such information could serve to clarify the results of past studies and allow the design of future experiments that definitively identify the contributions of NHEs.

Objectives of this thesis

The goal of this thesis in general is to examine some mechanisms of solute homeostasis in two interesting fishes at multiple levels of biological organization and to incorporate the techniques of molecular physiology to address some outstanding questions.

In the study of the Pacific hagfish, my objectives were the following:

1) to examine the novel possibility of inorganic phosphate uptake across the isolated skin and gill pouches,

2) to measure the kinetics of phosphate uptake by the skin, gill, and gut and to investigate the mechanism of transport, and

3) to clone and sequence the phosphate transporter from the hagfish gill.

In the study of the North Pacific spiny dogfish, *Squalus suckleyi*, my specific objectives were as follows:

4) to examine the ability of the dogfish to acclimate to reduced salinity and

5) to detail the time course of changes in solute homeostasis over an ecologically relevant short time scale.

To examine some of the dogfish's homeostatic mechanisms on the molecular level, I then set out to study dogfish NHEs. Accordingly, my goals for this project were:

6) to clone and sequence the full-length dogfish NHE2 and NHE3 from the gill,

7) to create cell lines that stably express each transporter in active form, and

8) to determine effective concentrations of inhibitory drugs for dogfish NHE2 and NHE3.

Table 1.1. Composition of seawater (SW), freshwater (FW), and intracellular fluid and blood plasma from hagfish and dogfish. Totals exceed the sum of listed constituents because of the presence of unmeasured solutes. μ, μmol l^{-1} levels; 0, undetected; -, not reported. Data from Bedford (1983a), Currie and Edwards (2010), DOE (1994).

Constituent	**Concentration (mmol kg water^{-1})**						
	SW (35‰ salinity)	**Brackish water (21‰ salinity)**	**FW (general)**	***Eptatretus stoutii* muscle**	***Eptatretus stoutii* plasma**	***Squalus acanthias* muscle**	***Squalus acanthias* plasma**
Sodium	469	281	0.3	132	549	53	268
Potassium	10	6	0.04	144	11	115	5
Calcium	10	6	0.4	2.6	5	8	8
Magnesium	53	32	0.15	17.5	19	11	5
Chloride	546	327	0.2	107	563	62	248
Phosphate	μ	μ	μ	86.4	5	10	11
Bicarbonate	2.2	1.5	1	-	3	-	4
Urea	0	0	0	1.5	2.8	320	376
TMAO	0	0	0	211	0	150	90
Amino acids	0	0	0	71	<1	225	10
Total	1090	650	1	992	1155	-	987

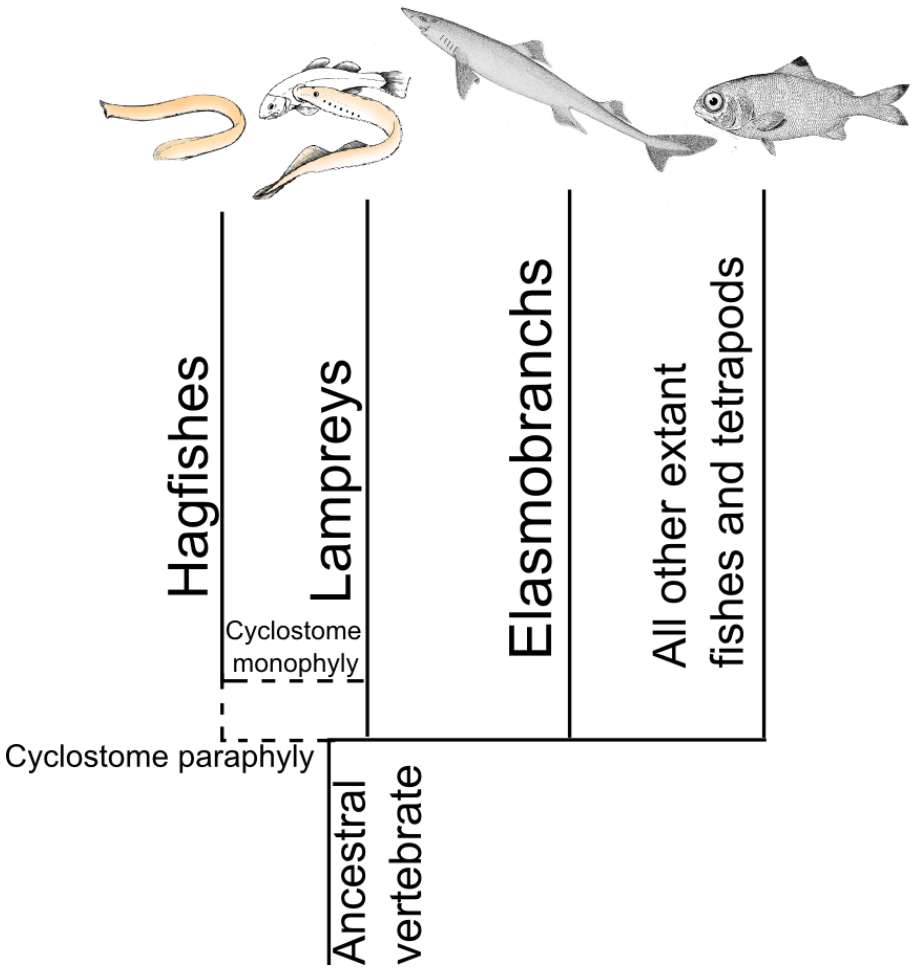

Figure 1.1. Phylogenetic relationships among the study organisms. Hagfish diverged from the vertebrate lineage around 500 million years ago, and elasmobranchs diverged from the remainder of the vertebrate lineage around 400 million years ago. The monophyly of the cyclostome clade containing hagfishes and lampreys is debated. Left two images ©1997 Philippe Janvier and licensed for use under the Creative Commons Attribution License - Version 3.0. Third image from *The Fisheries and Fisheries Industries of the United States* by G. Brown Goode (1887) and fourth image from *Oceanic Ichthyology* by G. Brown Goode and Tarleton H. Bean (1896) (copyrights expired).

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Chapter II

Mechanisms of phosphate absorption in the Pacific hagfish

(*Eptatretus stoutii*)

Introduction

Inorganic phosphate (P_i) is an essential nutrient for all organisms. It functions as a pH buffer, is critical in cellular metabolism as the active part of the energy carrier, ATP, and is the major component of bone mineral (Tenenhouse 2007). To obtain P_i, specialized transport systems in animals allow for efficient uptake of P_i against electrochemical gradients. In higher vertebrate species, the intestine plays the primary role in the acquisition of P_i from the diet. In marine environments, concentrations of P_i are extremely low, ranging from <0.1 to 3 μmol P_i kg^{-1} of water (Rafferty 2011). However, P_i levels in muscle and plasma have been measured at 86.4 mmol kg^{-1} and 5.0 mmol kg^{-1}, respectively, in the Atlantic hagfish, *Myxine glutinosa* (Currie and Edwards 2010). Therefore, the hagfish and other aquatic organisms must possess efficient mechanisms to obtain P_i and maintain homeostasis.

The hagfishes (Phylum Chordata, Class Myxini) form the most ancient group of living craniates and may represent the oldest living connection to the ancestral vertebrate (Bardack 1998). Consequently, when studying the evolutionary development of traits in vertebrates, hagfish present an important model organism. Presently, there is very little knowledge of the digestive physiology of hagfish. Hagfish have no true stomach and both digestion and absorption are suggested to occur in the intestine (Glover et al. 2011b). The intestine has also been reported to be a site for other physiological roles including immune (Uzzell et al. 2003) and endocrine functions (Thorndyke and Falkmer 1998). Also unlike other fishes, the gills of hagfish occur as rows of six to eight

pairs of internal pouches that run laterally along the body. Water is drawn in through a central nostril, which distributes water to each gill pouch via an afferent water duct. Water flows countercurrent to the blood that is within vessels of the pouch wall before exiting through an efferent water duct. This efficient countercurrent exchange system provides a favorable surface for exchange of ions, gases, and waste products between the blood and water (Mallatt and Paulsen 1986). The gills are, therefore, suggested to play an important role in ionoregulation, acid-base balance and nitrogenous waste excretion (Braun and Perry 2010; Choe et al. 1999; Tresguerres et al. 2006).

Hagfishes are osmoconformers, allowing their internal body osmolarity to closely match that of the external marine environment. It has been postulated that this could accommodate much greater skin permeability than in other vertebrates (Glover et al. 2011a). This could allow increased nutrient absorption during the rare and specialized feeding events of the hagfish. In nature, hagfish live on the seafloor and feed mostly on carrion such as dead fishes, sharks, and whales (Tamburri and Barry 1999). Notably, hagfish burrow into the carrion and feast from within (Bardack 1998; Martini 1998). Conditions within the decomposing carcass expose the hagfish to high concentrations of organic and inorganic nutrients, including $P_{i,}$ and present an opportunity for the hagfish to absorb nutrients directly across the skin and gills. This novel mode of nutrient uptake was recently confirmed by Glover et al. (2011a), who demonstrated that *E. stoutii* can absorb the amino acids alanine and glycine across gill and skin epithelium. The possibility for a vertebrate animal to absorb major inorganic macronutrients such

as P_i from the environment has never been examined. We therefore hypothesized that hagfish utilize similar uptake mechanisms for other macronutrients important for growth and development. This hypothesis was investigated using *ex vivo* techniques to determine the primary mechanisms of P_i uptake by the Pacific hagfish.

Materials and Methods

Animals

Pacific hagfish *Eptatretus stoutii* (Lockington) were caught using baited traps from Barkley Sound (Vancouver Island, Canada). Hagfish were transported by boat to Bamfield Marine Sciences Centre (BMSC) and maintained in 5,000 L tarpaulin-covered outdoor tanks supplied with flow-through seawater at 9-12 °C. Animals were not fed at any time following collection and were used for experimentation within 4 weeks of capture. For RNA isolation, tissues were dissected, snap frozen in liquid nitrogen, and held at -80 °C. The experiments were conducted during August, 2011, and August, 2012, and all procedures were approved by the Animal Care Committees of BMSC and the University of Alberta.

Solutions

All salines were prepared without Ca^{2+} and Mg^{2+} in order to minimize formation of insoluble phosphate salts. The standard Ca^{2+} and Mg^{2+}-free HF saline contained NaCl, 490 mmol l^{-1}; KCl, 8 mmol l^{-1}; $NaHCO_3$, 41 mmol l^{-1}; glucose, 5 mmol l^{-1}; pH=7.8 (Glover et al. 2011a). Sodium dependent phosphate uptake was tested using a Na^+, Ca^{2+}, and Mg^{2+}-free HF saline ($C_5H_{14}NOCl$ (choline chloride), 490 mmol l^{-1}; KCl, 8 mmol l^{-1}; $KHCO_3$, 41 mmol l^{-1}; glucose, 5 mmol l^{-1}; pH=7.8) (Glover et al. 2011a). To test for an inhibitory effect of phosphonoformic acid (PFA), PFA was added to Ca^{2+} and Mg^{2+}-free HF saline at a concentration of 10 mmol l^{-1}. PFA is a structural analogue of pyrophosphate

and is a known inhibitor of the NaPi-IIb transporter (slc34a2 in HUGO nomenclature) in mammals (Loghman-Adham 1996). Phosphate solutions for concentration-dependent uptake experiments were made by spiking the standard saline with a 100 mmol l^{-1} NaH_2PO_4 working stock in Ca^{2+} and Mg^{2+}-free HF saline or with a 100 mmol l^{-1} KH_2PO_4 working stock in Na^+, Ca^{2+}, and Mg^{2+}-free HF saline as appropriate. The following concentrations of P_i were tested for intestine and skin: 0.25, 0.5, 1.0, 2.0, 5.0 and 10 mmol l^{-1}. In the gills the following concentrations were tested: 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 5.0 and 10 mmol l^{-1}.

Intestinal P_i flux measurements

Hagfish were euthanized in 5 g l^{-1} neutral-buffered tricaine methanesulfonate (TMS; AquaLife, Syndel Laboratories Ltd, Vancouver, BC, Canada) and the entire gastrointestinal tract was dissected from the animal and flushed with hagfish saline (see *Solutions*). The portion of intestinal tract between the bile duct and cloacal region was divided into eight sections of 2-4 cm in length. A pilot study suggested slightly higher mean P_i uptake in the anterior regions of the intestine compared to the posterior region, but the variance between groups was comparable to the variance within groups, and the difference was not statistically significant. To ensure that there was no influence of regional uptake differences, intestinal segments were systematically rotated across all treatments.

Intestinal P_i flux measurements were conducted using a modified gut sac method previously described for hagfish (Glover et al. 2011b). In brief, each

intestinal section was formed into a sac by ligating one end with suture thread and inserting into the other end a ~5 cm length of flared cannula (PE-50 tubing; Intramedic, Clay-Adams, Parsippany, NJ, USA) secured in place with suture thread. Ca^{2+} and Mg^{2+}-free hagfish (HF) saline (see *Solutions*) containing [^{32}P]-orthophosphoric acid radionuclide (6 μCi per mole P_i; Perkin-Elmer, Boston, USA) was injected into the gut sac via the cannula until the sac was firm but pliable to the touch and the cannula was sealed with a sewing pin or by heat sealing. The gut sac was immersed in 10 ml of aerated Ca^{2+} and Mg^{2+}-free HF saline containing unlabeled P_i for a 2 h flux period (Fig. 2.1 a). In all experiments, tissues were symmetrically exposed on each side to solutions of identical composition, excepting that only one side contained radiotracer P_i amongst the unlabeled P_i. At the end of the period, the gut sac was drained and cut open laterally. No radiolabeled P_i was detected in the saline on the serosal side. To remove materials bound to the mucus layer, the mucosal surface was gently scraped with a glass microscope slide to remove the mucus layer and was then rinsed three times with isotope displacement solution (200mmol l^{-1} Na_2HPO_4 in Ca^{2+} and Mg^{2+}-free HF saline) to displace any isotope potentially adsorbed to the surface. The intestinal section was then stretched across graph paper for determination of surface area.

Skin P_i flux measurements

Skin P_i flux measurements were conducted using a modified method previously described for hagfish by Glover et al. (2011a). In brief, modified flux

chambers were constructed from 20 ml plastic scintillation vials with a circular hole of 2.835 cm^2 area cut out of the screw-top lids. Two small holes were drilled in the bottom of the chamber to serve as ports for the sample and for an air line. Several patches of skin (~3cm x ~3cm) were dissected from the anterior half of the euthanized animal dorsal to the level of the branchial pores. Patches were placed over the top of the vial and secured in place by the lid with the external surface of the skin facing inside the vial. The chamber was inverted and placed into a container holding 20 ml of aerated Ca^{2+} and Mg^{2+}-free HF saline containing unlabeled P_i (Fig. 2.1 b). Ten milliliters of Ca^{2+} and Mg^{2+}-free HF saline containing [^{32}P]-orthophosphoric acid radionuclide was then injected into the skin chamber through the sample port and an airline (PE 50 tubing) was inserted to mix and aerate the solution. Skin flux measurements were run for a 2 h period and following this, skin was removed from the chambers, scraped with a glass microscope slide, and rinsed three times with isotope displacement solution (200mmol l-1 Na_2HPO_4 in Ca^{2+} and Mg^{2+}-free HF saline). No radiolabeled P_i was detected in the saline on the serosal side. Uptake by the skin was expressed per unit surface area exposed (nmol cm^{-2} h^{-1}). Pilot experiments revealed no difference in P_i uptake between more anterior and more posterior sections of the skin, but to eliminate this possibility, skin segments were systematically rotated across all treatments.

Gill perifusion

Phosphate uptake across the gills of hagfish was investigated using a modified *ex vivo* gill perifusion method previously described by Glover et al. (2011a). Gill pouches were dissected from euthanized hagfish and the afferent and efferent water ducts of each pouch were cannulated with flared PE50 tubing that was secured in place with surgical silk (Fig. 2.1 c, d). Initial trials were conducted using food coloring dissolved in hagfish saline to test the efficacy of the preparation and validate perifusion of the branchial water channels. Two to three milliliters of Ca^{2+} and Mg^{2+}-free HF saline was injected through the afferent water duct into the gill pouch to exchange water and expel any trapped air. The pouch was immersed in 10 ml of aerated Ca^{2+} and Mg^{2+}-free HF saline containing unlabeled P_i. The afferent cannula of each gill pouch was connected to a peristaltic pump and the gill was perifused with Ca^{2+} and Mg^{2+}-free HF saline (osmotically matched to the external saline using mannitol) containing [^{32}P]-orthophosphoric acid radionuclide (6 μCi per mole P_i) at a rate of 6.0 ml h^{-1} for 3 h. Perifusates were collected over 30 min intervals. Note that matching the osmolality of solutions to within 1 mOsm/kg is critical in this application because any osmotic gradient between the internal and external saline causes rapid movement of water through the epithelium and consequent dilution or concentration of the internal saline. Glover and colleagues previously stated that a flow rate of 5.9 ± 1.1 ml h^{-1} was ideal for perifusing hagfish gill pouches and prevented pouch swelling, cannula clogging and development of artifact transport pathways (Glover et al. 2011a). At the completion of the experiment, gill pouches

were disassembled, blotted dry and weighed. To calculate P_i uptake, the initial 30 min perifusate fraction was discarded and then the disappearance of isotope from each 30 min fraction after that (representing the final 2.5 h of perifusion) was determined based on the difference in radioactivity between the afferent and efferent solutions. The uptake of P_i in each of the five final periods was averaged, divided by gill wet weight and converted to an hourly rate.

Tissue digestion and radioisotope analysis

Intestine and skin samples were digested in 1 N HNO_3 for 48 h at 60°C. Scintillation fluid (ACS, Amersham Biosciences, Baie d'Urfe, Quebec, Canada) was added to digests and samples were then held in the dark for 12 h before counting on a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA). Manual quench correction was employed for intestine and skin digests by generating quench curves over a range of tissue masses.

Reverse-transcriptase PCR

Tissues (intestine, gill, skin and kidney) from un-manipulated animals were dissected, snap frozen in liquid nitrogen and stored at -80 °C. RNA was isolated from each tissue using TRIzol Reagent (Life Technologies, Carlsbad, California), treated with DNAse I and purified using RNeasy Mini spin columns (Qiagen Canada, Montreal, Quebec) according to the manufacturers' instructions. RNA was then converted to cDNA using 1 μg total RNA through the Fermentas reverse transcriptase reaction (Thermo Fisher Scientific, Waltham, Massachusetts). A partial sequence of a NaPi-II-like (slc34a2-like) transcript was

discovered in a hagfish gill Illumina® transcriptome database acquired by our laboratory (via BGI, Shenzhen, China), and the following PCR primers were constructed: 5' to 3', GTCATCAACACAGACTTCCC and GTAAAGGACGGCAAACCAAC. Standard RT-PCR was performed using the following cycling parameters: 95 °C for 2 minutes followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. Aliquots of each PCR reaction were subjected to electrophoresis on a 2% agarose gel in 1X sodium boric acid buffer at 5 V cm^{-1} for 50 minutes, stained with ethidium bromide. The amplified 393 bp fragment was sequenced and was most similar to the NaPi-IIb (slc34a2) from *Xenopus tropicalis* and *Takifugu rubripes* (84 and 79% amino acid identity, respectively), according to a BLASTp search of the NCBI non-redundant protein database.

Cloning of full-length cDNA

From the initial fragment identified in the transcriptome database, several rounds of 5' and 3' Rapid Amplification of cDNA Ends (RACE) were employed. Using the cDNA template created from DNase I-treated RNA from hagfish gill, Takara 5' and 3' RACE and SMARTer 5' and 3' RACE (Clontech Laboratories, Mountain View, California) were alternately employed to amplify several overlapping sequences to span the full coding sequence (CDS) up to the 5' and 3' untranslated regions. Finally, a full-length transcript was amplified with Phusion polymerase (New England Biolabs, Ipswich, Massachusetts) using the following primers and PCR cycling conditions: 5'-ATGCAATCACCAACCACTGAG-3'

and 5'-TCTGTTGAGAGCAGTGAGCC-3' at 98 °C for 1 minute followed by 40 cycles of 98 °C for 10 seconds, 61 °C for 30 seconds, and 72 °C for 65 seconds. The product was then cloned into the pXT7 vector (provided by Dr. Warren Gallin). The plasmid was sequenced and the full insert sequence was identical to the deduced CDS.

Multiple sequence alignment and phylogenetics

A BLASTp search of the NCBI nonredundant protein database revealed high sequence identity to other NaPi-IIb (slc34a2) sequences. Selected NaPi transporter sequences were aligned using MUSCLE and curated using GBLOCKS with less stringent parameters. Maximum likelihood phylogenetic analysis was conducted using the PhyML algorithm in the program SEAVIEW, using the LG substitution model with the gamma distribution of the across site rate variation optimized by the program.

Data presentation and statistical analysis

All data are presented as means ± SEM. Differences between groups were evaluated using one-way ANOVA followed by Dunnett's *post-hoc* test. Regression models were created from mean values using SigmaPlot 11.0 and tested for significance using the extra-sum-of-squares F test. Differences were considered statistically reliable at $p < 0.05$.

Results

P_i uptake measurements

Initial experiments using ^{14}C-inulin showed no radioactivity in the intestinal tissue digests or skin digests (results not shown), confirming that material did not adhere to the surface or diffuse into the tissue and that the scraping technique was effective at removing surface bound mucus. Therefore we considered all ^{32}P activity in tissue digests as corresponding to P_i uptake. No ^{32}P radioactivity above background levels was detected in serosal salines, indicating minimal transepithelial transfer of ^{32}P during the experiment. No significant differences in P_i uptake were detected among different regions of the hagfish intestine or among different regions of skin.

The rate of P_i uptake by the hagfish intestine and skin was dependent on the external concentration of P_i from 0.25 to 10 mmol l^{-1} (Fig. 2.2). P_i uptake rates in the intestine were linear with increasing P_i concentration, indicating predominance of a non-saturable uptake pathway. P_i uptake rates in the skin were linear at high P_i concentration, but at P_i concentrations $\leq$ 2 mmol l^{-1} there appeared to be a second, saturable uptake pathway that exhibited classical Michaelis-Menten kinetics (Fig. 2.2 b inset). The Michaelis-Menten kinetic model provided a significantly better fit than a linear model by the extra-sum-of-squares F test ($p<0.01$). A three-parameter (sigmoid) Hill equation did not provide a significantly better fit than the two-parameter Michaelis-Menten model ($p>0.9$). The estimated apparent affinity constant (K_m) for the saturable P_i uptake in the skin was 0.93 ± 0.5 mmol l^{-1}.

The rate of P_i uptake by the hagfish gill also depended on the external concentration of P_i (Fig. 2.3). As in the skin, P_i uptake rates in the gill were also consistent with two parallel transport pathways, so further experiments were conducted at lower P_i concentrations (0.10 and 0.05 mmol l^{-1}). At concentrations < 2 mmol l^{-1}, P_i uptake included a component that exhibited saturable kinetics (Fig. 2.3 b). The data can be fit to either a two-parameter simple Michaelis-Menten model or a three-parameter Hill equation. The three-parameter Hill equation provides a significantly better fit. The estimated apparent K_m, V_{max}, and Hill coefficients for the two kinetic models are given in Table 1. Discounting the lowest two P_i concentrations does not significantly alter the fit of either model.

P_i uptake by the intestine and skin at 2 mmol l^{-1} P_i was not dependent on external sodium and was not inhibited by 10 mmol l^{-1} PFA (Fig. 2.4 a and b). In gill (Fig. 2.4 c), PFA resulted in a non-significant reduction in P_i uptake by 23%, while the absence of Na^+ reduced uptake by 25%; again this was not statistically significant ($p > 0.05$).

Molecular biology

Using reverse transcriptase PCR, a partial sequence of a NaPi-IIb-like transcript was detected in the intestine, skin, gill, and kidney of the hagfish (Fig. 2.5). The predicted amino acid sequence of this fragment was most similar to the NaPi-IIb protein (HUGO gene nomenclature: slc34a2) from *Xenopus tropicalis* and *Takifugu rubripes* (84 and 79% amino acid identity, respectively).

Using 5' and 3' RACE, a full coding sequence was identified and cloned from hagfish gill cDNA. A BLAST search of the NCBI non-redundant protein database matched the full deduced amino acid sequence most closely to the NaPi-IIb (slc34a2) proteins from *Chelonia mydas*, *Anolis carolinensis*, and *Xenopus laevis* (61%, 59%, and 67% sequence identity, respectively) (Table 2.2). The cDNA and deduced amino acid sequences of the full-length transporter are shown in Figure 2.6.

Alignment of the amino acid sequences of several NaPi-II transporters with the hagfish NaPi-II revealed strong conservation in certain regions of the sequence (Fig. 2.7). In particular, one residue reported to be critical for transport function (L403 in hagfish NaPi-II) is conserved across hagfish NaPi-II and vertebrate NaPi-IIb proteins. Phylogenetic analysis indicates that the cloned sequence indeed encodes an NaPi-II transporter, and that the hagfish NaPi-II is earlier-diverging than, and/or ancestral to, the vertebrate NaPi-II transporters (Fig. 2.8).

Discussion

This study shows the remarkable ability of several epithelial tissues of the hagfish to absorb P_i from the environment. Thus the hagfish may be able to obtain this major inorganic nutrient in large amounts during rare encounters with high P_i concentrations, such as when feeding within seafloor carrion. To the best of our knowledge, the present report is the first conclusive demonstration of P_i uptake by the skin and gill of any chordate animal. In addition, while we have cloned a partial sequence for the NaPi-IIb-like (slc34a2) transporter for hagfish, there also appears to be a non-saturable (at the tested concentrations) P_i uptake pathway present in these tissues.

Intestinal absorption

In the hagfish intestine, the rate of P_i uptake increased linearly with increasing P_i concentrations. This linear relationship strongly suggests that the non-saturable transport component of P_i uptake predominates in the hagfish intestine across the range of P_i concentrations tested. Similar non-saturable P_i uptake has been reported in the intestine (Avila et al. 2000) and pyloric caeca (Sugiura and Ferraris 2004) of rainbow trout, the only fish studied in this regard. However, in both of these studies, carrier-mediated active P_i uptake was also observed. Similar non-saturable (postulated to be diffusive and/or passive in previous studies) and saturable P_i uptake processes have been reported in the intestine of rats (Berner et al. 1976) and rabbits (Danisi and Straub 1980). We suggest that active transport of P_i in the hagfish intestine may be occurring, but its

contribution is masked by the high rate of transport through the non-saturable pathway.

The hagfish has been previously examined with respect to the intestinal absorption of amino acids (Glover et al. 2011b) and the transport of glucose by erythrocytes (Young et al. 1994). Both of these studies suggested that hagfish nutrient transport systems are more similar to those of mammals than to those of teleost fishes. With regard to intestinal P_i uptake, the hagfish appears to be similar to both trout and mammals (Danisi and Murer 2010), primarily due to the high P_i concentrations presumably present in the intestine after feeding. However, further physiological and molecular characterization is required to confirm this.

Absorption by skin and gill

We have demonstrated for the first time in any chordate or vertebrate that the skin and gill of the hagfish are able to absorb significant amounts of P_i directly from the aqueous medium. Absorption of major inorganic macronutrients such as P_i across extra-intestinal epithelia is rare in animals, with quantitative measurements of P_i uptake from the environment being limited thus far to only two species of bivalve mollusk, the American oyster, *Crassostrea virginica* (Pomeroy and Haskin 1954) and mussel, *Mytilus edulis* (Ronkin 1950). In both of these studies, P_i accumulation was detected in the gills of the mollusk, however, these researchers did not investigate the uptake mechanism. Inorganic phosphate uptake has also been suggested to occur across the integument of amphibians, with Mobjerg and colleagues (2007) identifying a strong positive correlation

between the P_i concentration in the aquatic medium and P_i concentrations in urine and lymph of the toad, *Bufo bufo*. However, the present study is the first to quantitatively measure P_i uptake by extra-intestinal epithelia.

Inorganic phosphate uptake across both skin and gills of hagfish increased linearly with increasing external P_i concentrations above 1.0 mmol l^{-1}. This suggests a non-saturable uptake pathway at P_i concentrations above 1.0 mmol l^{-1}, similar to that observed in the intestine. Hagfish, therefore, appear to have the remarkable capacity to obtain large amounts of P_i across extra-intestinal epithelia when they opportunistically encounter high environmental P_i concentrations, such as those present within a decomposing animal. This could provide immediate access to this important nutrient even before digestion and absorption proceed within the gut.

Interestingly, the skin and gill also both exhibited saturable P_i transport at P_i concentrations below 1.0 mmol l^{-1}. The simple Michaelis-Menten two-parameter models showed apparent K_m values for P_i uptake in the skin and gill (0.93 ± 0.5 and 0.56 ± 0.4 mmol l^{-1}, respectively). However, these estimated K_m values are based on only a few data points, and measurements at additional P_i concentrations would reduce uncertainty in the estimates. In addition, the saturable kinetics observed in the skin and gill were similar to those reported for mammalian and trout intestine active P_i transport systems, which generally have an apparent K_m from 0.6-1.3 mmol P_i l^{-1} (Avila et al. 2000; Berner et al. 1976; Danisi and Straub 1980; Sugiura and Ferraris 2004). It is also notable that a sigmoidal, three-parameter model best described the P_i uptake kinetics in the

hagfish gill. Such observations are more difficult to interpret for intact tissues than for individual isolated transporters, as there may be various processes at work. Sigmoidal uptake kinetics have also been observed for hagfish skin and gill absorption of amino acids (Glover et al. 2011a). Those authors attributed such kinetics to multiple transport pathways operating in concert. For that study and the present, the sigmoidal kinetics could also be attributed to one or more instances of positive cooperativity. Sigmoidal kinetics are commonly assumed to indicate cooperative binding of substrate through modulation of the individual enzyme of interest (Weiss 1997). When working with intact tissues, the possibility arises that cells may adjust their transport mechanisms based on the environment to which they are exposed. In the case of the hagfish gill, it is conceivable that the usual capacity for active P_i uptake is low, but becomes stimulated upon exposure to a nutrient-rich environment. Such ability would be adaptive in an animal that usually encounters minuscule levels of external P_i, but encounters high levels during occasional feeding events.

The contributions of the skin and gill epithelia to total P_i uptake are considerable. For a hagfish of average size exposed to 2 mmol P_i l^{-1}, a rough approximation from the data suggests that the total P_i uptake across all gills would be about 2600 nmol h^{-1}, while that across the entire skin surface would be about 3500 nmol h^{-1}. The magnitude of intestinal uptake is certainly underestimated in the present study because the scraping procedure removes much of the mucosal cell layer; however the observed rate can be taken as a minimum and would supply at least 200 nmol h^{-1} across the entire gut.

Possible active transport mechanisms

The most common P_i transporters detected in fish and mammalian absorptive epithelia are in the NaPi-II family (HUGO nomenclature: slc34) (Avila et al. 2000; Kohl et al. 1996; Sugiura 2009; Werner and Kinne 2001). These transporters preferentially import one HPO_4^{-2} with three Na^+, though some are capable of coupling P_i uptake to H^+ import in the absence of Na^+, and they generally exhibit an apparent K_m for P_i between 30 and 250 μmol l^{-1} and an apparent K_m for Na^+ of around 40 mmol l^{-1} (Sugiura and Ferraris 2004; Werner and Kinne 2001). Currently, two NaPi-IIb isoforms (NaPi-IIb1 and NaPi-IIb2) have been identified in the intestine and kidneys of elasmobranchs and teleosts (Sugiura 2009; Werner and Kinne 2001), while only one NaPi-IIb and two divergent NaPi-IIa (slc34a1) and NaPi-IIc (slc34a3) transporters have been identified in tetrapod tissues (Bergwitz and Juppner 2012; Virkki et al. 2007). Using reverse transcriptase PCR, we detected the presence of an NaPi-IIb-like fragment in hagfish intestine, skin, gill, and kidney. NaPi-IIb in mammals and NaPi-IIb1 in fishes have been demonstrated to play a significant role in P_i uptake in the intestine. We therefore investigated the role of the hagfish NaPi-IIb-like transporter in P_i uptake using a pyrophosphate structural analogue and putative NaPi-II transport blocker, PFA (Loghman-Adham 1996).

In the hagfish intestine and skin, P_i uptake at 2 mmol l^{-1} was not inhibited by 10 mmol l^{-1} PFA and was not affected by a nominally Na^+-free medium, demonstrating that transport systems other than NaPi-IIb predominate at this P_i concentration. In the gill, however, PFA reduced average P_i uptake by 23%,

while the absence of Na^+ reduced uptake by 25%, consistent with the involvement of an NaPi-IIb transport system. The observation of Na^+-independent P_i uptake in the intestine and skin does not exclude the involvement of the NaPi-IIb-like transporter we identified in the intestine, as multiple isoforms exist and some have been shown to be capable of Na^+-independent P_i uptake most likely coupled to H^+ cotransport (Sugiura and Ferraris 2004). However, other transporters may be active in addition to the NaPi-IIb-like transporter in the hagfish intestine and skin.

In the hagfish gill, results from RT-PCR, Na^+-dependence, PFA susceptibility, and kinetic studies all suggest the involvement of the NaPi-IIb-like transporter in P_i uptake. The apparent K_m in the three-parameter model was 280 μmol P_i l^{-1}, which is close to the range observed for other NaPi-IIb transporters expressed in *Xenopus* oocytes (Werner and Kinne 2001). Furthermore, the partial inhibition of P_i uptake caused by PFA or Na^+-free conditions indicate that the NaPi-IIb-like transport system may not be the only P_i uptake system in the hagfish gill, though it appears to contribute to active P_i uptake. Other conceivable routes of P_i uptake could be via the Pit [slc20] family of sodium-phosphate cotransporters, via H^+-coupled cotransport through the NaPi-II or Pit transporters, or possibly via exchange for bicarbonate or organic anions through an anion exchanger protein (Wehrle and Pedersen 1989).

Sequence analysis of the hagfish NaPi-II transporter

The full CDS cloned from the gill shares 67% amino acid identity with *X. tropicalis* NaPi-IIb (slc34a2). In the few amino acid residues that are known to be functionally important for NaPi-II transporters, we have found no striking

differences between the hagfish NaPi-II and vertebrate NaPi-II transporters. One residue critical for transport activity, N182 in the hagfish NaPi-II, is conserved across all examined NaPi-II transporters (Köhler et al. 2002). Another residue implicated in Na^+ affinity that differs between vertebrate NaPi-IIa and NaPi-IIb, L403 in hagfish NaPi-II, is conserved across hagfish NaPi-II and vertebrate NaPi-IIb proteins. The hagfish NaPi-II may thus be called an NaPi-IIb-like transporter. Phylogenetic analysis indicates that this transporter is possibly ancestral to the vertebrate NaPi-II (slc34) transporters, as its apparent divergence predates the divergence of the vertebrate NaPi-IIa and NaPi-IIb forms. Therefore, it appears likely that the NaPi-IIb type transporter arose before the NaPi-IIa type. This is the first P_i transporter cloned from the hagfish, and the existence of multiple hagfish NaPi-II transporters remains a possibility. Functional expression of the hagfish NaPi-II transporter in *Xenopus* oocytes may in the future be used to examine the Na^+ dependence and mechanism of transport of this protein to determine whether the observed P_i uptake in the nominal absence of Na^+ could have proceeded through this transporter.

Conclusion

In summary, we have demonstrated that the hagfish has the remarkable ability to absorb the major inorganic nutrient P_i into the gill and skin. This is the first quantitative measurement of P_i uptake by the gill or skin of any chordate/vertebrate species and provides further insight into the adaptations of hagfish to maximize absorption of nutrients across multiple surfaces during opportunistic encounters with high nutrient concentrations. Finally, P_i absorption in hagfish gill may occur in part via an NaPi-IIb-like transport system, and we have cloned the full cDNA for an NaPi-IIb-like transporter that may be involved.

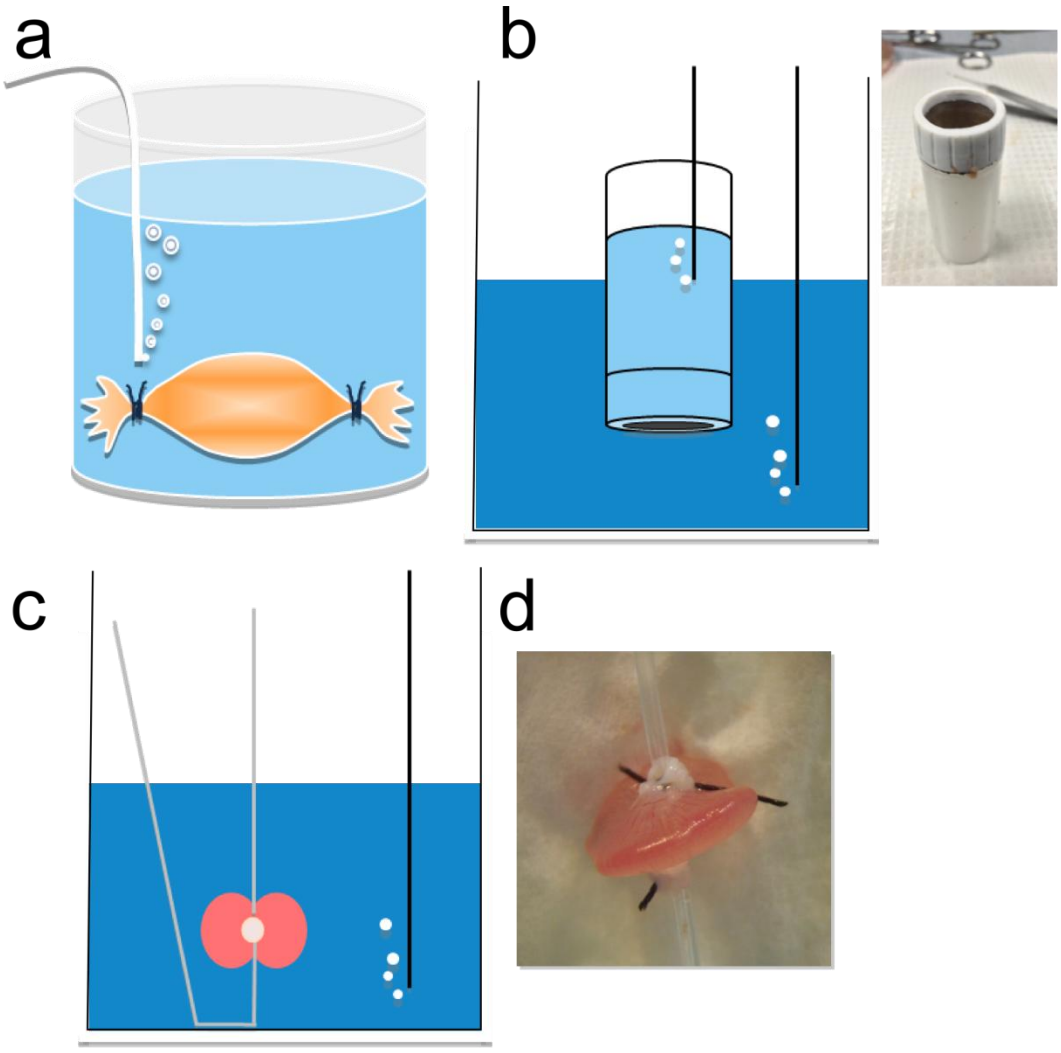

Figure 2.1. Depiction of the organ bath preparations used. a) Gut sac preparation. b) Skin preparation. Inset shows digital image of skin chamber preparation. c) Perifused gill preparation. d) Digital image of a perfused gill pouch. Images a, b, d courtesy of Dr. Aaron Schultz. Inset b courtesy of Alex Clifford.

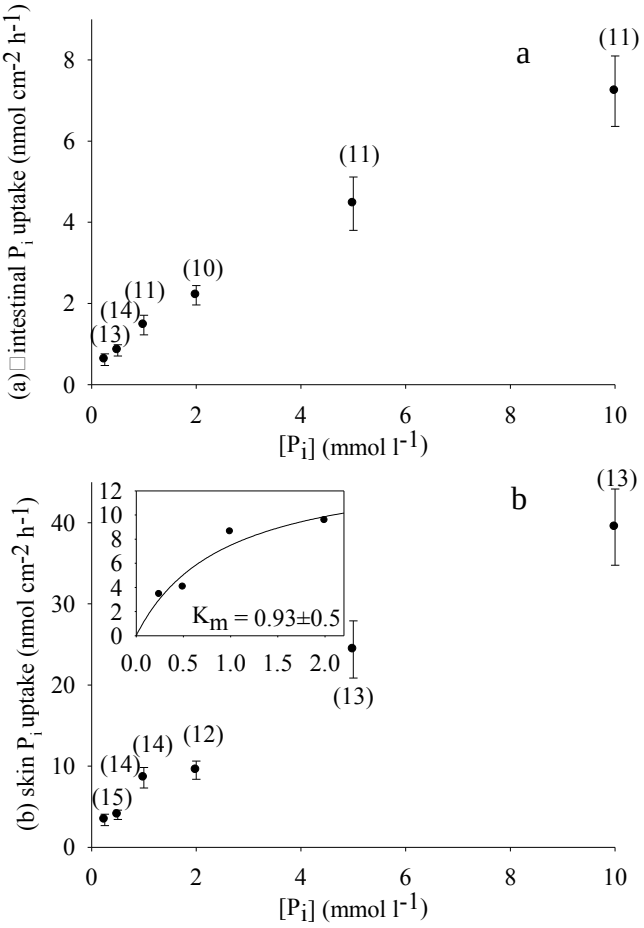

Figure 2.2. Concentration-dependent P_i uptake (nmol cm^{-2} h^{-1}) in the a) intestine and b) skin of the hagfish. Uptake rates in the intestinal gut sacs were linear with increasing $[P_i]$, indicating predominance of a passive uptake pathway. Uptake rates in the skin, using modified Ussing chambers, were linear at high $[P_i]$, with a second transport pathway that conformed to Michaelis-Menten kinetics at low $[P_i]$ (inset). Values represent mean ± SEM and sample sizes are indicated in parentheses. The curve inset was fitted using SigmaPlot v11.0.

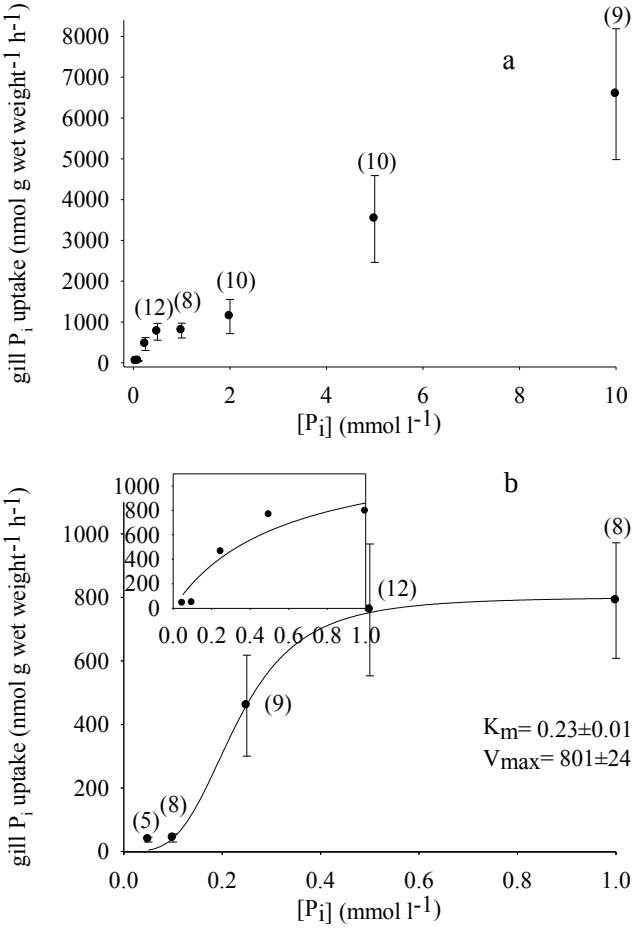

Figure 2.3. Concentration-dependent P_i uptake (nmol g^{-1} h^{-1}) in isolated perfused hagfish gill pouches. a) P_i uptake rates at $[P_i]$ ranging from 0.05 to 10 mmol l^{-1}. b) Focused view of P_i uptake rates at low $[P_i]$ (0.05 to 1 mmol l^{-1}). Uptake rates in the gill were linear at high $[P_i]$, while a saturable transport pathway was clearly visible at low $[P_i]$. Values represent the mean ± SEM and sample sizes are indicated in parentheses. The sigmoidal curve in (b) was fitted using SigmaPlot v11.0, and revealed an apparent K_m of 0.23 ± 0.01 mmol l^{-1}. Inset shows alternative fitting of data to simple Michaelis-Menten kinetics; the Hill equation provided a significantly better model for the observed values (see Table 2.1).

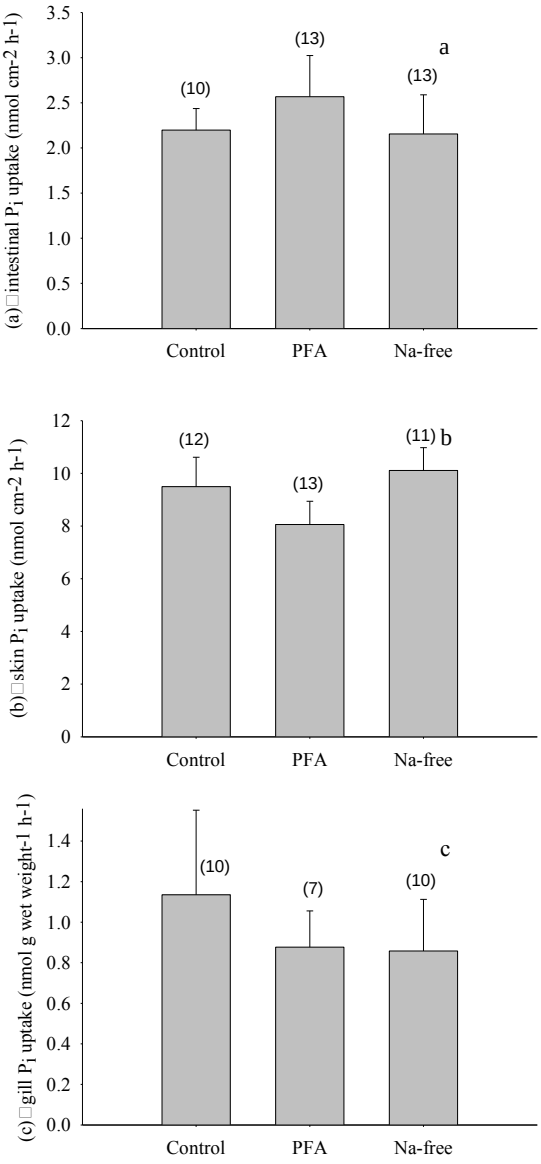

Figure 2.4. Potential effect of phosphonoformic acid (PFA; 10 mmol l^{-1}) and Na^+-free solutions on P_i uptake in the a) intestine, b) skin, and c) gill of hagfish. Potential inhibition of P_i uptake by PFA and dependence of P_i uptake on external Na^+ were tested in each tissue at a $[P_i]$ of 2 mmol l^{-1} and were compared to control uptake rates at the same $[P_i]$. Bars indicate mean ± SEM and sample sizes are indicated in parentheses. No statistically significant differences were detected ($p > 0.05$, ANOVA).

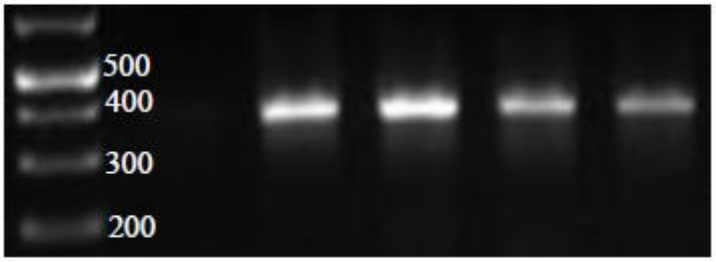

Figure 2.5. Expression of hagfish NaPi-IIb-like mRNA in different tissues determined by reverse transcription-polymerase chain reaction (RT-PCR) experiments. Reactions were performed using NaPi-IIb specific primers and amplification of a 393-bp partial sequence was detected using reverse-transcribed RNA from intestine, skin, gill, and kidney. Lane 1: size markers, Lane 2: no template control, Lane 3: intestine, Lane 4: gill, Lane 5: skin, Lane 6: kidney. Size markers are indicated at left.

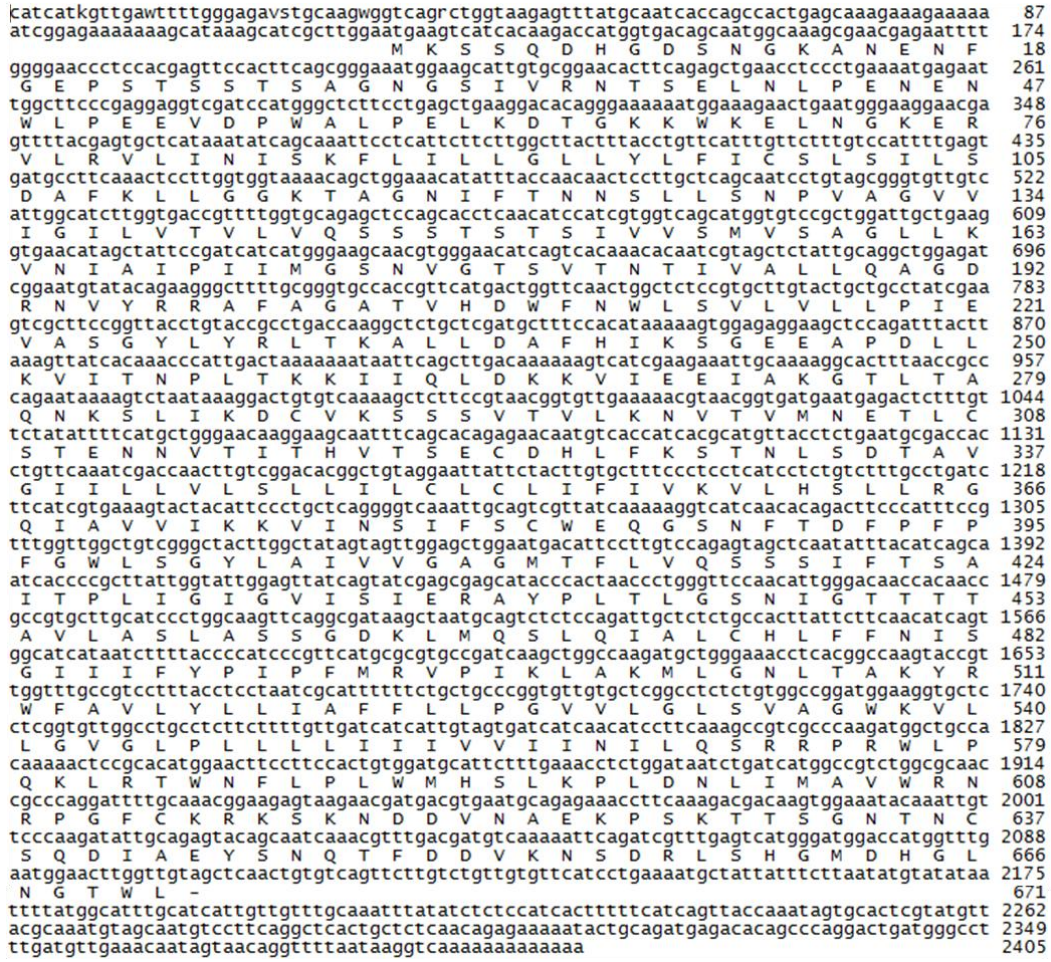

Figure 2.6. Nucleotide and deduced amino acid sequences of the cloned hagfish NaPi-IIb-like transporter. The 2492 bp cDNA contains a 2016 bp open reading frame encoding 671 amino acids followed by a 269 bp 3' UTR including a poly-A tail and preceded by a 5' UTR of at least 120 bp that contains stop codons in all three reading frames.

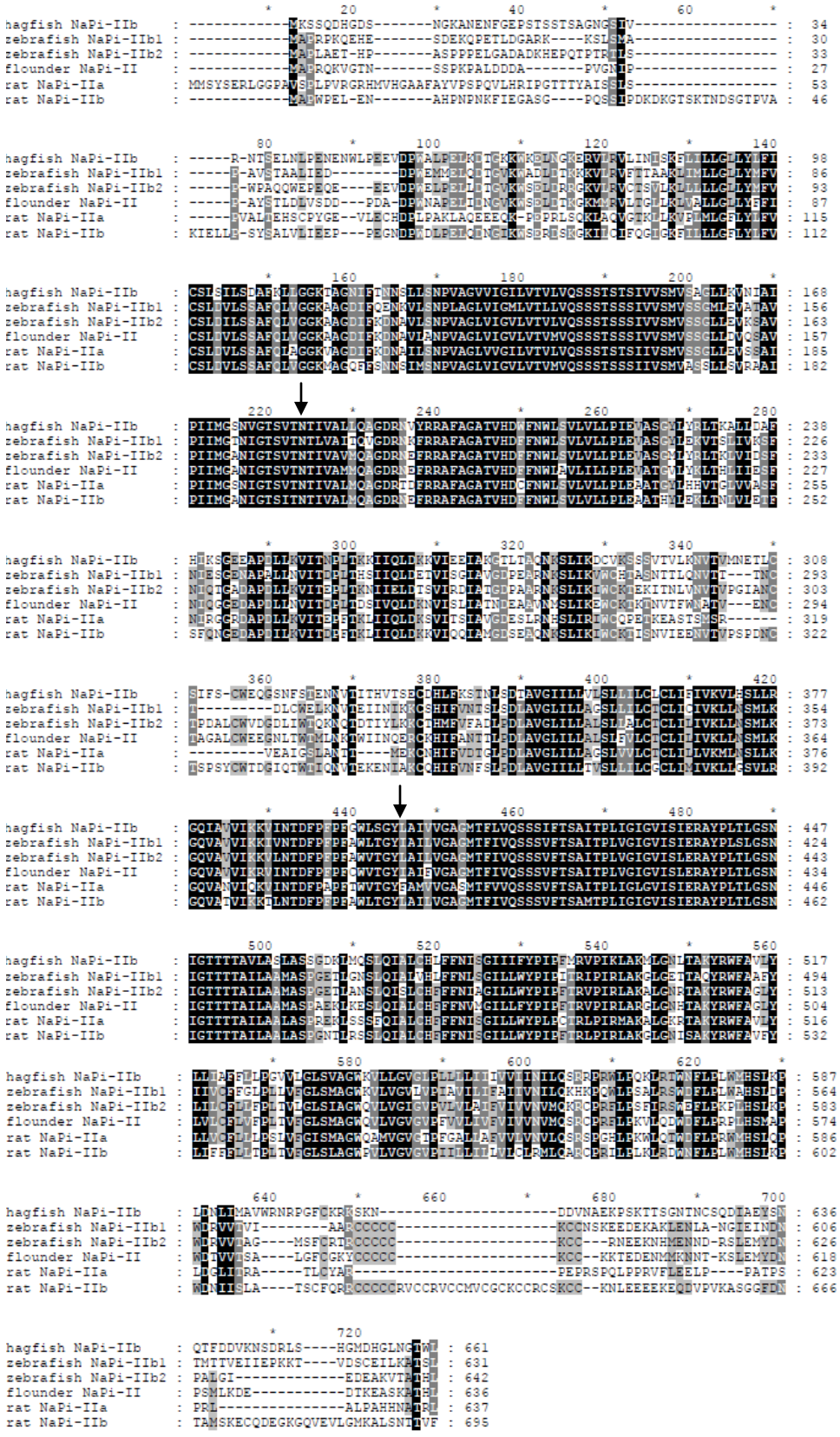

Figure 2.7. (previous page) **MUSCLE alignment of deduced amino acid sequences of select NaPi-II transporters.** Residues highlighted in black are strongly conserved and residues in grey are moderately conserved. The arrows indicate two residues reported to affect sodium dependence and transport mode (Graham *et al.* 2003, Kohler *et al*. 2002). Accession numbers: zebrafish NaPi-IIb1 AAH96858.1, zebrafish NaPi-IIb2 NP_878297.1, flounder NaPi-II AAB16821.1, rat NaPi-IIa NP_037162.1, rat NaPi-IIb NP_445832.1.

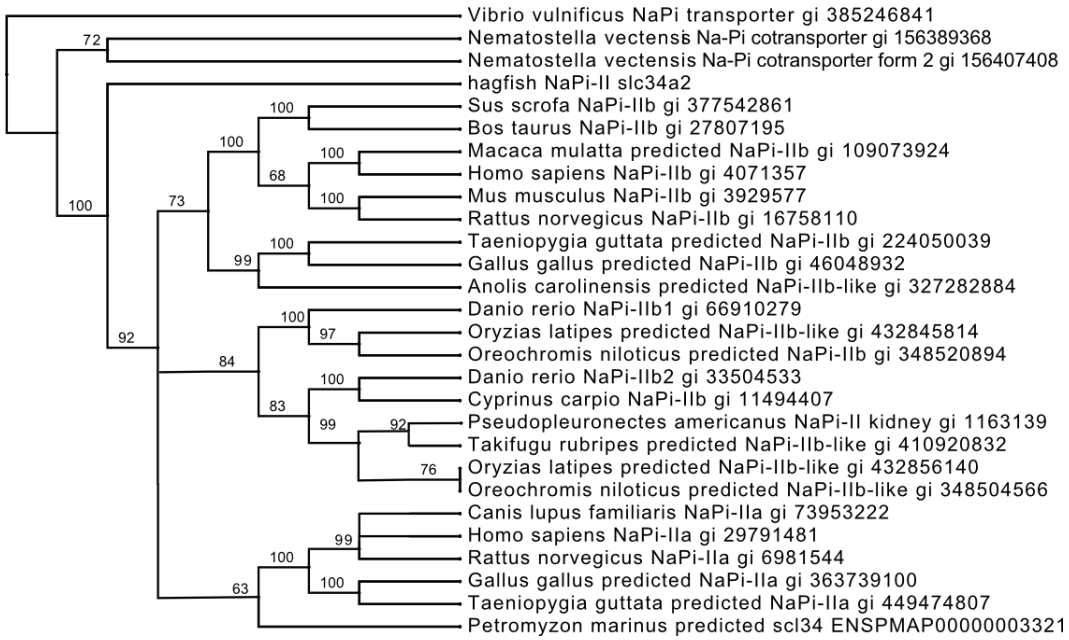

Figure 2.8. Maximum Likelihood phylogenetic tree of various NaPi-II transport proteins. Numbers indicate the percentage of 300 bootstrap replicates that supported that separation. Branches with less than 60% bootstrap support have been collapsed. The tree indicates that the cloned hagfish NaPi-II transporter is earlier diverging than, and/or ancestral to, the vertebrate NaPi-II transporters. Genbank identification numbers are given at the end of the protein name, except for the *P. marinus* protein, which was retrieved from Ensembl.

Table 2.1: **Regression models of gill high affinity P_i uptake kinetics.** Abbreviations (units): K_m, apparent affinity constant (mmol l^{-1}); V_{max}, estimated maximal transport velocity (nmol g^{-1} h^{-1}); HC, Hill coefficient; Adj R^2, adjusted R^2; SSR, sum of squared residuals; sig., significance of additional parameter above the simpler model. The 3-parameter model provides a significantly better fit than the 2-parameter model ($p<0.01$) by the extra-sum-of-squares F test. Parameters shown are best estimate ± standard error.

Model	**K_m**	**V_{max}**	**HC**	**Adj R^2**	**SSR**	**sig.**
linear	-	-	-	0.673	543721	-
2-parameter	0.56±0.4	1347±544	-	0.8665	54433	-
3-parameter	0.23±0.01	801±24	3.5±0.6	0.9955	1232	$p<0.01$

Table 2.2. Amino acid sequence comparison of the cloned hagfish NaPi transporter with those of other organisms

Species	Protein	ID No.	Identity
Nematostella vectensis	NaPi cotransporter	XP_001634963.1[a]	59%
Xenopus laevis	NaPi cotransporter	AAF21135.1[a]	67%
Chelonia mydas	NaPi-IIb	EMP37379.1[a]	61%
Anolis carolinensis	NaPi-IIb-like	XP_003226172.1[a]	59%
Danio rerio	NaPi-IIb1	AAH96858.1[a]	62%
Pseudopleuronectes americanus	NaPi-IIb kidney	AAB16821.1[a]	57%
Latimeria chalumnae	NaPi-IIb	ENSLACP 00000014496[b]	66%
Squalus acanthias	NaPi-IIb2 kidney	AAG35796.1[a]	63%
Homo sapiens	NaPi-IIb	NP_006415.2[a]	64%

[a]GenBank accession No., [b]Ensembl Protein ID No.

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Chapter III

Time course of the acute response of the

North Pacific spiny dogfish shark (*Squalus suckleyi*) to low salinity[1]

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Introduction

The spiny dogfish species *Squalus suckleyi* and *S. acanthias* are known to enter estuaries and are occasionally found in salinities less than 25‰ (McMillan and Morse 1999). However, these sharks cannot survive long-term in very dilute brackish water environments. Although these two species are very closely related, they may exhibit differences in their tolerance to brackish water. Several elasmobranch species are able to thrive in estuaries and even freshwater [FW] environments (reviewed in Ballantyne and Fraser (2012)). The bull shark, *Carcharhinus leucas,* for example, normally resides in seawater [SW] but has been found in very dilute FW including 4,000 km up the Amazon River (Thorson 1972) and in the FW Lake Nicaragua (Thorson 1971). When euryhaline elasmobranchs move between SW and FW, important physiological reorganization occurs in hormone levels, organ and tissue function, cellular organization, and protein expression (Dowd et al. 2010; Hazon et al. 2003; Piermarini and Evans 2001). For instance, an elasmobranch moving from SW to FW must adjust its concentration of solutes, primarily Na^+, Cl^-, and urea, and also deal with a large osmotic water load imposed by the FW environment. Eventually, the salt-secreting rectal gland is deactivated and salt-gaining strategies are activated, either through ingestion of food (Haywood 1973; Wood and Bucking 2010) or increased absorption of salts across the gills (Bentley et al. 1976).

The closely related spiny dogfish sharks, *Squalus acanthias* in most ocean basins, *Squalus suckleyi* in the North Pacific (Ebert et al. 2010), have been

demonstrated to tolerate dilute SW for short periods. In a test of *S. acanthias* exposed to 24-28‰ salinity for three to nine days, plasma osmolality fell by 10-15% and plasma Cl^- fell by 9%, but the fish appeared healthy in such an environment for at least nine days (Burger 1965). Another experiment exposing *S. acanthias* to a gradual dilution to 25‰ salinity over six days further revealed a 15% drop in plasma Na^+ and a 30% drop in plasma urea (Bedford 1983). In contrast, when *S. acanthias* is transferred to full FW or to artificial media lacking Na^+ or Cl^-, it loses equilibrium and dies within one to three hours (Dakin 1908). However, euryhaline species in FW are able to curtail measurable chloride efflux (Smith 1931a), maintain blood Na^+ and Cl^- levels steady at around 30% less than in SW-acclimated individuals, and maintain urea levels steady at around 50% less than in SW-acclimated individuals (Piermarini and Evans 1998; Pillans et al. 2006; Pillans et al. 2004; Smith 1931a; Smith 1931b). Thus a primary difference between stenohaline and euryhaline elasmobranchs has been suggested to be the ability of the latter to maintain plasma solute homeostasis by reducing solute efflux and activating solute uptake mechanisms (Evans 1984).

The present investigation assesses the time course of potential acclimation of North Pacific spiny dogfish (*S. suckleyi*) to dilute SW [60% SW, 21‰ salinity]. 21‰ salinity was chosen because the Na^+ concentration at this salinity is approximately equal to the Na^+ concentration in the blood plasma. The rate of acclimation is an ecologically relevant problem that has been previously unstudied in this commonly studied shark. Special regard has been given to the change in plasma solutes as well as the measured rates of urea efflux and oxygen

consumption. It was hypothesized that the dogfish is capable of some degree of acclimation to dilute SW, and it was therefore predicted that the dogfish would initially exhibit high rates of solute loss that would decline over time as the animal acclimated to the new environment.

Materials and methods

Animals

North Pacific spiny dogfish, *Squalus suckleyi* (Ebert et al. 2010), were caught by hook and line from the Trevor Channel (Vancouver Island, BC, Canada) and immediately transferred to the Bamfield Marine Sciences Centre, where they were held in a 151,000 liter circular tank provided with flowing seawater (9-13°C, 31-33‰ salinity). Fish were fed a ration of hake equal to 2% of body weight every four days; before experimentation, fish were fasted for four days. All experimental protocols were approved by the University of Alberta and Bamfield Marine Sciences Centre animal care committees.

Surgery

Dogfish were anaesthetized with neutral buffered MS-222 (0.2 g L^{-1}) (Syndel Laboratories, Qualicum Beach, BC), and were each fitted with a cannula into the caudal artery following the protocol described previously (Tresguerres et al. 2005). After surgery, fish were placed into opaque 40 liter experimental boxes with flowing seawater to recover for 24 hours. To test the effect of surgery on urea efflux, two groups of fish did not undergo surgery, but were placed directly into boxes for a 24 hour acclimation period. The rates of urea efflux were not significantly different between non-cannulated (n=4) and cannulated (n=11) animals and therefore results were combined.

Low salinity exposure

After a 24 h acclimation period, urea fluxes were measured over periods of one to three hours using an intermittent flow protocol as described by Wood et al. (2007). After measurement of initial solute fluxes in control SW (t = -1 to 0 h; 32‰ salinity, 900 mOsm kg^{-1}), the inflowing water was replaced with a premixed and aerated blend of SW and FW (21‰ salinity, 590 mOsm kg^{-1}). Complete water exchange was accomplished within ten minutes. Salinity was monitored using a refractometer and later precisely measured by osmometry. The boxes were flushed several times with new water or resupplied with flowing water at times (t)=0, 6, 9, 12, 18, 24, 36, and 48 h. Even over the longest static period, total ammonia concentration did not rise above 36 μmol l^{-1} [0.6 ppm]. At all times, a flowing SW bath maintained the water temperature at 9-13°C. A concurrent group of fish was held in 100% SW for 48 h as a control for confinement stress.

Blood sampling and analytical procedures on plasma samples

The average blood volume of *S. suckleyi* is 6.8% of body weight (Thorson 1958), or approximately 70-170 ml for the range of dogfish used in this experiment. Blood was withdrawn via the caudal artery cannula (600-900 μL samples) at times (t)=0, 2, 4, 6, 9, 12, 18, 24, 36, and 48 hours post-transfer to 21‰ salinity. The sampled volume was replaced with an equal volume of heparinized (100 i.u. ml^{-1}) dogfish saline containing, in mmol L^{-1}: NaCl 280.0, KCl 6.0, $CaCl_2$ 5.0, $MgCl_2$ 3.0, Na_2SO_4 0.5, Na_2HPO_4 1.0, $NaHCO_3$ 4.0, Urea 350.0, trimethylamine oxide 70.0, and glucose 5.0 at pH=7.8. Blood pH was

immediately measured by an Orion ROSS Micro pH electrode (Fisher Scientific, Ottawa, ON), with samples thermojacketed at 10°C. The blood samples were then centrifuged at 12,000 *g* for 2 minutes to obtain plasma; 40 µl of plasma were used for total CO_2 determination using a Corning model 965 total CO_2 analyzer. Measured values of blood pH and total CO_2 were used to calculate [HCO_3^-] and P_{CO2} at 10° C by the Henderson-Hasselbalch equation. Changing plasma ionic strength causes changes in CO_2 solubility and the apparent dissociation constant for carbonic acid, which were accounted for as described by Cooper and Morris (2004b). Remaining plasma and red blood cell pellets were then frozen separately at -80°C for later analysis. Plasma [Na^+] and [K^+] were measured using a Thermo Scientific model iCE 3300 Atomic Absorption Spectrometer. Plasma [Cl^-] was measured using a Buchler digital chloridometer. Plasma and water osmolality were measured using a Vapro vapor pressure osmometer. The urea content of plasma samples and water samples were measured by the method of Rahmatullah and Boyde (1980) modified for microplate and with plasma samples diluted 1:5000. Unless otherwise mentioned, all the reagents used in this study were purchased from Sigma (St. Louis, MO).

Measurement of urea flux and oxygen consumption

To measure rates of urea flux, 100 ml water samples were collected at times (*t)* = -1, 0, 2, 4, 6, 8, 9, 12, 17, 18, 22, 24, 34, 36, 46, and 48 hours after transfer to 21‰ salinity. Osmolality and urea concentrations were measured as described above. In addition, one group of non-cannulated animals was used to measure rates of oxygen consumption. The box was sealed by applying a tight-

fitting plastic-wrapped foam lid to the water surface (Wood et al. 2007). Samples were taken at the start and end of a 30 minute period, during which the P_{O2} declined by about 10%. Oxygen content was measured using a DOX P_{O2} electrode (Fisher Scientific, Ottawa, ON) thermojacketed at 10°C. The measured P_{O2} was converted into moles of dissolved oxygen using the solubility constants derived by Boutilier et al. (1984).

Data presentation and statistical analysis

All data are presented as means ± SEM. Data were analyzed with one-way repeated measures ANOVA and individual time periods were compared to mean control values using the Holm-Sidak *post-hoc* test. For data that were not normally distributed, a repeated measures ANOVA on ranks was used with Dunnett's *post-hoc* test. In all figures, asterisks (*) or plus signs (+) indicate $p < 0.05$ for the comparison indicated.

Results

All Pacific dogfish sharks survived surgery, and after 48 hours of exposure to 21‰ salinity, one of 15 sharks appeared moribund. This animal was euthanized and data from that shark at *t*=36 and 48h discarded. After 48 h of exposure, average body weight of the dogfish was 11.6±0.6% greater than initial body weight (n=12). Accordingly, all of the calculated rates in this report are standardized to the initial body weight of each animal.

No disturbance in the pH of arterial blood was evident during 48 hours of exposure to diluted seawater [21‰ salinity]. Blood pH averaged between 7.88 and 7.97 (Figures 3.1 and 3.2). Transient increases in plasma total CO_2 were detected at *t*=6 and 12 h, with values reaching 5.78±0.33 and 5.61±0.40 mmol l^{-1}, respectively, compared to control values of 4.30±0.23 mmol l^{-1} (n=8-9) (Fig. 3.1). Total CO_2 comprises dissolved CO_2 gas and HCO_3^-, as well as a small amount of CO_3^{-2} at high pH values. Resting blood P_{CO2} values averaged 0.153±0.010 kPa at *t*=0 h, but reached as high as 0.22 kPa at *t*=6, 12, and 24 h after exposure to 21‰ salinity (Fig. 3.2). After 36 hours, P_{CO2} was not significantly different from control levels. HCO_3^- comprised the majority of total CO_2, ranging from 4.25±0.26 milliequivalents l^{-1} at *t*=0 h to 5.70±0.32 milliequivalents l^{-1} at *t*=6 h, and as low as 3.33±0.36 milliequivalents l^{-1} at *t*=36 h after exposure to 21‰ salinity (n=7-9) (Fig. 3.2).

As shown in Figure 3.3, the initial rate of O_2 consumption (*t*=-1 to 0 h) in non-cannulated sharks was equal to 1355 μmol kg^{-1} h^{-1}, in agreement with Wood

et al. (2007). After the first four hours of exposure, the rate of O_2 consumption increased and was significantly higher at t=12 h, reaching 158% of control O_2 consumption (Fig. 3.3). After this time, the rate of O_2 consumption gradually declined to control values.

The concentrations of the major plasma ions at each sampling time are shown in Figure 3.4. Total osmolality of the blood plasma decreased from 939±9 mOsm kg^{-1} at t=0 to 769±8 mOsm kg^{-1} at t=48h (n=7-9). This decrease became more gradual over time, appearing to asymptotically approach a value near 760 mOsm kg^{-1}, well above that of the aqueous medium (590 mOsm kg^{-1}). Plasma [Cl^-] decreased from 238±2 mmol L^{-1} at t=0 to 221±2 mmol L^{-1} at t=9 h and did not change significantly after this time (n=7-9). Plasma [Na^+] significantly decreased from 221±3 mmol L^{-1} at t=0 to 170±4 mmol L^{-1} at t=48 h (n=6-9), whereas plasma [K^+] showed no significant changes, with mean values ranging from 6.3±0.2 mmol L^{-1} to 8.3±0.5 mmol L^{-1} (n=7-9) (Fig. 3.4).

The concentration of urea in the plasma (t=0, 284±18 mmol L^{-1}) dropped sharply after four hours by about 15% and continued to decline throughout, reaching 188±14 mmol L^{-1} at 48 hours post-transfer (n=7-9) (Fig. 3.5A). The flux of urea out of the animal was determined over each time period by measuring appearance of urea in the water. Observed rates of urea efflux were not different in cannulated and non-cannulated fish, so the data were combined. The initial rate of urea efflux in 100% SW was 186±52 μmol kg^{-1} h^{-1} in the pre-exposure control period. The rate of urea efflux in 100% SW did not change significantly over 48 hours (not shown). After transfer to 21‰ salinity, the rate of urea efflux

significantly increased to 443±30 μmol kg^{-1} h^{-1} during the *t*=0-2 hour period. Urea flux continued to increase significantly to a maximum of 762±75 μmol kg^{-1} h^{-1} at *t*=34-36 hours (n=14-15) (Fig. 3.5B).

Discussion

North Pacific spiny dogfish exposed to low salinity are capable of surviving at least 48 hours in 21‰ salinity. However, osmotic cataracts as described by Siezen (1988) had formed by the end of the exposure. The increased body weight of the dogfish post-transfer was likely a result of osmotic water influx that was not fully compensated within 48 hours. Winter skate, *Leucoraja ocellata*, exposed to 17.5‰ salinity do not exhibit compensation in net water gain until three to four days post-transfer (Sulikowski et al. 2003). Similarly, the lip shark, *Hemiscyllium plagiosum*, exposed to 12‰ salinity exhibits similar weight gain compensation starting after two days (Chan and Wong 1977). Euryhaline bull sharks, *Carcharhinus leucas*, are able to acclimate to full FW and can effectively compensate for the osmotic water load and maintain similar body fluid volumes in SW and FW (Thorson 1962). However, studies vary widely in their lengths of acclimation and few illustrate the time course of these changes. As reviewed by Anderson et al. (2007), a return to normal pre-exposure weight is observable in most elasmobranchs exposed to dilute SW. It is possible that compensation of the water gain in dogfish sharks might occur after a longer exposure but the time course and degree of compensation remain to be determined.

The pH of the blood was not appreciably affected by exposure to dilute SW. This is not surprising given that elasmobranchs are able to quickly recover from perturbations in systemic pH homeostasis by employing acid-base transfer mechanisms at the gills (Heisler 1988; Tresguerres et al. 2005; Tresguerres et al.

2006; Tresguerres et al. 2010; Wood et al. 1995; Wood et al. 2007). The true blood acid/base and CO_2 status is best represented by a Davenport diagram (Fig. 3.2). During exposure to dilute SW the P_{CO2} of dogfish blood temporarily increased by 40%, concurrent with an observed increase in metabolic rate. This increase in CO_2 was concomitantly counteracted by increased blood [HCO_3^-], preventing variations in blood pH. Thus the respiratory acidosis was balanced by a metabolic alkalosis. These patterns are consistent with previously published reports (Cooper and Morris 2004a,b), in which the partially euryhaline shark *Heterodontus portusjacksoni* exposed to 26‰ and 17.5‰ salinity also exhibited increased plasma CO_2 from *t*=6-12 hours.

An increased rate of O_2 consumption was evident from 6-18 hours of exposure to dilute SW. This could indicate an avoidance response, a metabolic response, or both. In fact, Dowd et al. (2010) observed increased activity/movement in leopard sharks, *Triakis semifasciata*, exposed to 17.5‰ salinity. This was attributed to an initial avoidance response, suggesting that the sharks in nature would tend to swim out of the estuarine environment. An alternative explanation is that increased metabolism and activity upon encountering dilute SW could allow the sharks to complete activities such as hunting, feeding, or birthing pups (Castro 1993) in less time. The observed increase in O_2 consumption in our study could also be due in whole or part to increased metabolism and energy production specifically for dealing with the homeostatic challenges of dilute SW. Despite many attempts to calculate the energetic cost of osmoregulation and ionoregulation, reviewed by Evans (2008),

estimates vary from 0.5% to 11% of standard metabolic rate. However, it seems likely that the initial demands of reorganizing cells and tissues in response to different environments would create a significant, though temporary, increase in metabolism. In the present experiment, the temporary increase in O_2 consumption integrated from 6-18 hours, exclusive, amounts to an excess O_2 consumption of 5932 μmol kg^{-1}, or 539 μmol kg^{-1} h^{-1} above the control rate. This value is nearly 40% above the initial metabolic rate, suggesting that the temporary metabolic demands of reorganizing osmoregulatory systems may be large. However, increased physical activity may have contributed substantially to this increase in oxygen consumption. In the future, respirometry studies on fully euryhaline elasmobranchs such as the bull shark, *C. leucas*, or Atlantic stingray, *D. sabina*, will be informative in this regard.

Perturbations in the composition of blood plasma were evident. Plasma osmolality decreased, appearing to asymptotically approach a value near 760 mOsm kg^{-1}, distinctly hyperosmotic to the aqueous medium (590 mOsm kg^{-1}). Many studies have shown that this fall is mostly due to a reduction in plasma urea, with some loss of plasma sodium and chloride; a concomitant decrease in the intracellular concentrations of free amino acids and other osmolytes has also been demonstrated in *S. acanthias* (Ballantyne and Fraser 2012; Bedford 1983; Forster and Goldstein 1976; Piermarini and Evans 1998; Pillans et al. 2006). Plasma [Cl^-] decreased by about 9% during the first nine hours of exposure to dilute SW and was relatively stable thereafter. The initial loss appears to trigger compensatory mechanisms that succeed in stabilizing plasma [Cl^-] after around

six to nine hours. A similar change was observed in *S. acanthias* by Burger (1965), who reported that dogfish maintained steady plasma [Cl^-] for at least nine days post-transfer to dilute SW. Thus, regulatory mechanisms appear to decrease the efflux and/or increase the influx of Cl^- during low salinity exposure. Regarding efflux, the majority of Cl^- export likely occurs in the urine and rectal gland fluid. Measurements of urine and rectal gland fluid volumes and Cl^- concentrations have indicated that *S. acanthias* loses 6-12 mmol Cl^- kg^{-1} day^{-1} in SW and substantially more, around 18 mmol kg^{-1} day^{-1}, after acclimation to dilute SW, owing to the combination of increased urine flow and the maintenance of active rectal gland secretion (Burger 1965). Because dogfish maintain steady plasma [Cl^-] despite increasing Cl^- efflux, there must be a compensatory increase in Cl^- influx. Passive influx of Cl^- is still possible in 21‰ salinity, though the concentration gradient is substantially reduced. However, the fact that dogfish increase Cl^- influx despite a reduced concentration gradient points to active regulation of Cl^- uptake mechanisms. It has been demonstrated in the euryhaline *D. sabina* that the gills contain an slc26a4 pendrin-like Cl^-/HCO_3^- exchanger and that expression of this transport protein is inversely related to salinity (Piermarini and Evans 2002). This system likely also functions in the dogfish gill for acid-base regulation via HCO_3^- excretion and Cl^- uptake (Tresguerres et al. 2006) but could be involved in Cl^- homeostasis as well.

In contrast to Cl^-, plasma [Na^+] decreased and was not compensated within 48 hours. The initial 20% loss of Na^+ within the first 12 hours was for the most part stabilized, though plasma [Na^+] continued to decrease very slightly

throughout the exposure. In general, Na^+ efflux increases in dilute SW primarily due to increased losses through the urine and rectal gland fluid (Burger 1965). In SW-acclimated *S. acanthias*, estimates of total Na^+ efflux range from 15-20 mmol kg^{-1} day^{-1}, while Na^+ influx through the head end is 21-24 mmol kg^{-1} day^{-1} (Burger and Tosteson 1966). In waters of varying Na^+ concentration, Bentley et al. (1976) found that Na^+ influx in *Scyliorhinus canicula* displayed saturable kinetics, with influx exceeding efflux at and above Na^+ concentrations equivalent to 10‰ salinity. By measuring unidirectional fluxes as well as the transepithelial electrical potential across the gill, the same study also provided strong evidence that Na^+ influx occurs via active transport, probably utilizing a Na^+/H^+ exchange mechanism (NHE). NHE proteins have been found in elasmobranch gills and likely serve to eliminate acid and absorb Na^+ (Choe et al. 2005; Choe et al. 2007; Reilly et al. 2011; Tresguerres et al. 2005). Whether the dogfish is able to increase the activity of NHE in dilute SW is currently unknown.

The distribution of the osmotic water load in our study is uncertain. Chan and Wong (1977) found that the lip shark, *Hemiscyllium plagiosum*, behaves as a passive osmometer, *i.e.*, while plasma ion concentrations decrease as a result of water gain, the total amount of ions in the plasma remains relatively constant. The extent of haemodilution in the present study was not determined. However, if it is assumed that the water load (equal to 11% of body weight) is evenly distributed throughout total body water (72% of body weight for *S. suckleyi* (Thorson 1958)) and therefore led to at least a 15% dilution of the plasma, then the 9% decrease in plasma $[Cl^-]$ is less than expected, while the 20% decreases in

plasma [Na^+] and [urea] are greater than expected. This provides further evidence that Cl^- is well maintained, Na^+ is regulated after some time, and urea release is actively promoted.

Plasma urea concentrations sharply decreased between four and six hours and continued to decline gradually, while net urea efflux increased by 72% over the low salinity exposure. Interestingly, the rate of urea excretion in 21‰ salinity was significantly higher than in SW even during the first measurement period (443±30 μmol kg^{-1} h^{-1} at *t*=0-2 hours versus 186±52 μmol kg^{-1} h^{-1} during the pre-exposure control period) suggesting the ability to rapidly readjust to changes in salinity. In the same species, Wood et al. (1995) reported average urea excretion rates in SW equal to 225 μmol urea kg^{-1} h^{-1}, consistent with our measured rate. As reviewed by Hazon et al. (2003) and Perlman and Goldstein (1988), the flux of urea out of the animal appears to be due to increased renal clearance (Forster et al. 1972), in part due to decreases in urea transporter abundance in the kidney tubule (Yamaguchi et al. 2009). The usual rate of urea loss to the urine under control conditions can be estimated at less than 2 mmol kg^{-1} day^{-1} in *S. acanthias* (Hays et al. 1977). However, in *S. acanthias* exposed to 24‰ salinity, urine flow rate increases and fractional urea reabsorption decreases nearly 8-fold (Forster et al. 1972). In the present study, whole animal urea efflux rates amounted to 12-17 mmol kg^{-1} day^{-1}. Considered alongside previous reports, our data demonstrate that urea losses in the spiny dogfish species increase rapidly upon entry into dilute SW and also continue to increase over at least 48 hours, even though the gradient for loss is reduced, suggesting active regulation of the rate of urea loss.

Long-term survival in dilute SW?

Given the results observed, the question naturally arises as to whether *S. suckleyi* could survive indefinitely in a dilute SW environment. While *S. acanthias* exposed to a slightly more moderate dilution of 25‰ salinity SW can survive for at least nine days (Bedford 1983; Burger 1965), it was unclear whether dogfish can survive a greater dilution for extended periods. After 48 hours of exposure to a medium diluted by 40%, the dogfish in the present study appeared close to attaining a new steady state with reductions in plasma osmolality by 20%, plasma $[Na^+]$ by 20%, and plasma $[Cl^-]$ by 9%. In addition, plasma $[K^+]$, plasma CO_2, blood pH, and the final rate of oxygen consumption were not significantly different from pre-exposure levels. However, steady states were not attained with respect to plasma urea, total body water, or the rate of urea loss, suggesting that dogfish had not adequately acclimated to this hyposmotic stress.

Several studies have indicated that marine elasmobranch species require some minimum level of urea for normal body function. Yancey and Somero (1977) demonstrated that the muscle glycolytic enzyme LDH from several marine elasmobranch species requires physiological concentrations of urea for normal function. Siezen (1988) showed that reversible osmotic cataracts, also observed in the present study, form in the *S. acanthias* eye lens in the presence of low osmolality and less than 250 mmol l^{-1} urea. Also, Simpson and Ogden (1932) reported that the heart of *S. suckleyi* fails to beat when exposed to less than 200 mmol l^{-1} urea. After 48 hours of exposure to 21‰ salinity in the present study, plasma urea concentrations of the dogfish averaged only 188 mmol l^{-1}. This

would suggest that heart failure would be imminent with longer exposure to this condition. In fact, one dogfish was moribund at the end of the experiment, and some of the other dogfish that were returned to SW after 48 hours did exhibit delayed mortality over the following two weeks. Together, these suggest that 48 hours at 21‰ salinity is the maximum practical tolerance of this species to abruptly lowered salinity.

Because plasma [Na^+] and [Cl^-] reached new steady states, I propose that urea balance, specifically, the requirement for a minimum level of urea, is likely the limiting factor for survival of this species in diluted SW. The regulation of ion balance was apparently sufficient to stabilize plasma ion levels. However, the increased excretion of urea, while adaptive from the perspective of osmotic balance, apparently proceeded in an unsustainable manner that eventually jeopardized the health of the animals. Thus, dogfish do adjust their physiology in an attempt to acclimate to diluted SW, but the degree of this challenge probably precludes long-term survival in 21‰ salinity. Whether dogfish exposed to more moderately diluted SW for longer periods are able to curtail urea loss, modulate urea synthesis, and activate Na^+ uptake mechanisms provide ample opportunities for further investigation. Given that the barrier to euryhalinity appears to be a quantitative problem of influx versus efflux rates, determining whether the patterns observed in *S. suckleyi* and *S. acanthias* are reiterated in other marginally euryhaline species will help begin to construct a more general understanding of why euryhalinity is a successful strategy in some elasmobranch species but not others.

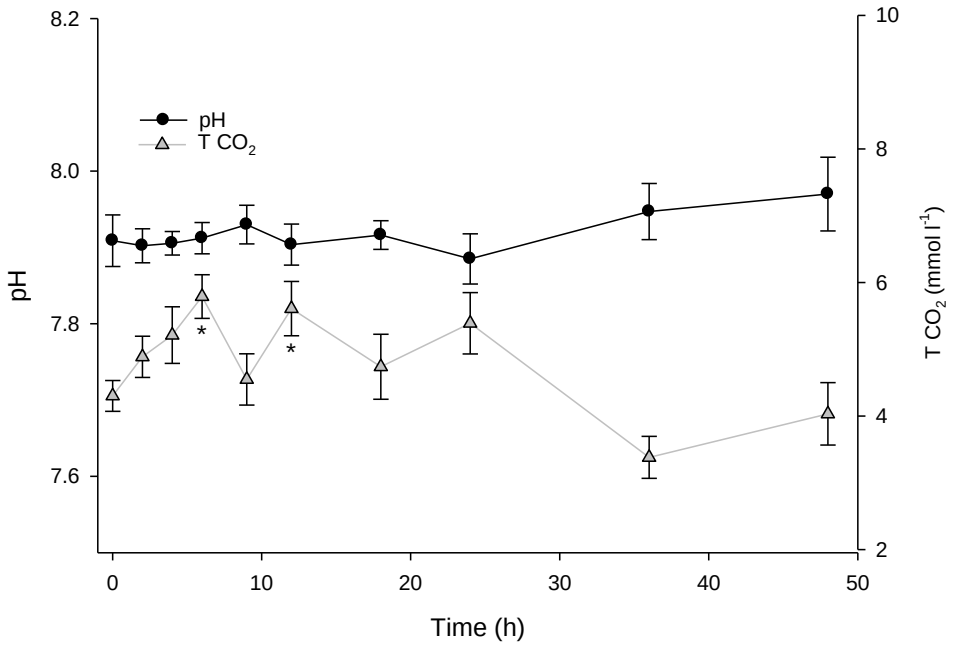

Figure 3.1. Arterial blood pH and plasma total CO_2 (mean±SEM) from sharks exposed to 21‰ salinity. pH (black circles, left ordinate axis) did not change appreciably over time (n=8-9). Total CO_2 (gray triangles, right ordinate axis) exhibited modest, transient elevations (n=7-9). Asterisks (*) indicate significant difference from control *t*=0 in 100% SW; $p<0.05$.

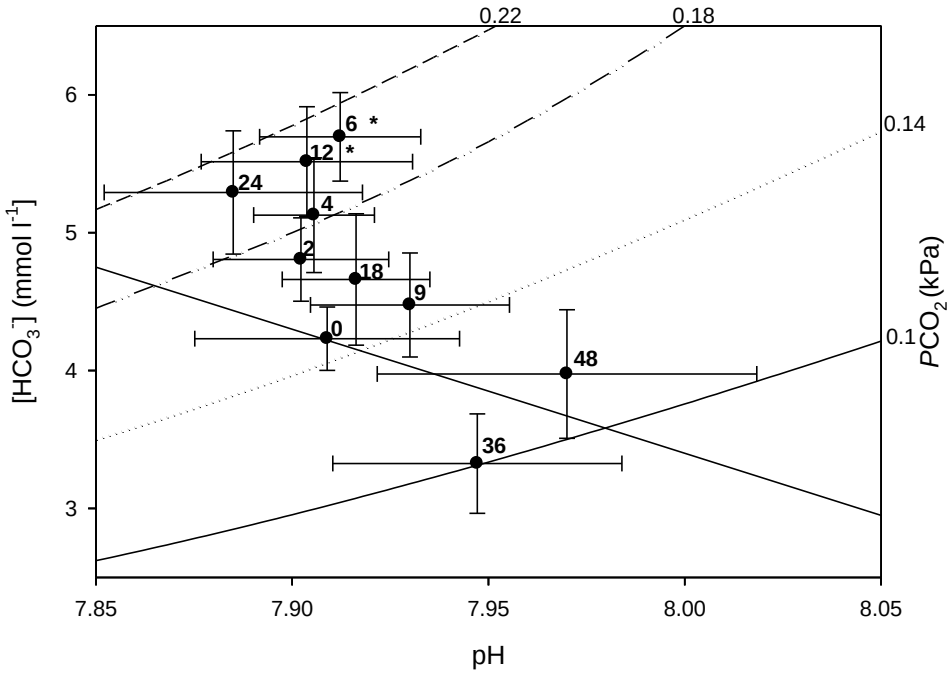

Figure 3.2. Blood pH/[HCO_3^-] diagram, or Davenport diagram (Davenport, H. W. 1974), depicting the acid-base status of the blood of sharks exposed to 21‰ salinity (means±SEM, n=7-9). A transient respiratory acidosis is compensated by increased retention of HCO_3^-. The solid straight line is the non-bicarbonate buffer line derived from experimental data using constants determined by Lenfant et al. (1966). Bolded numbers signify sampling time. Asterisks (*) indicate significant differences in pH and [HCO_3^-] from control *t*=0 in 100% SW; $p<0.05$.

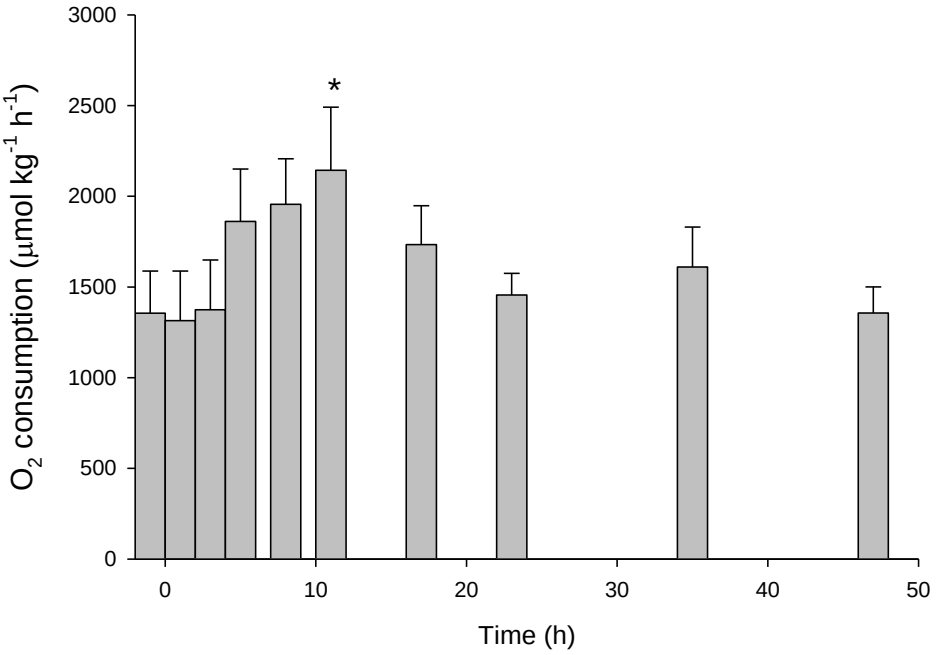

Figure 3.3. Oxygen consumption rates (mean+SEM) of non-cannulated sharks exposed to 21‰ salinity. A notable temporary increase in oxygen consumption was apparent between *t*=6-18h (n=4). Asterisk (*) indicates significant difference from control *t*=-1 to 0 hours in 100% SW; $p<0.05$.

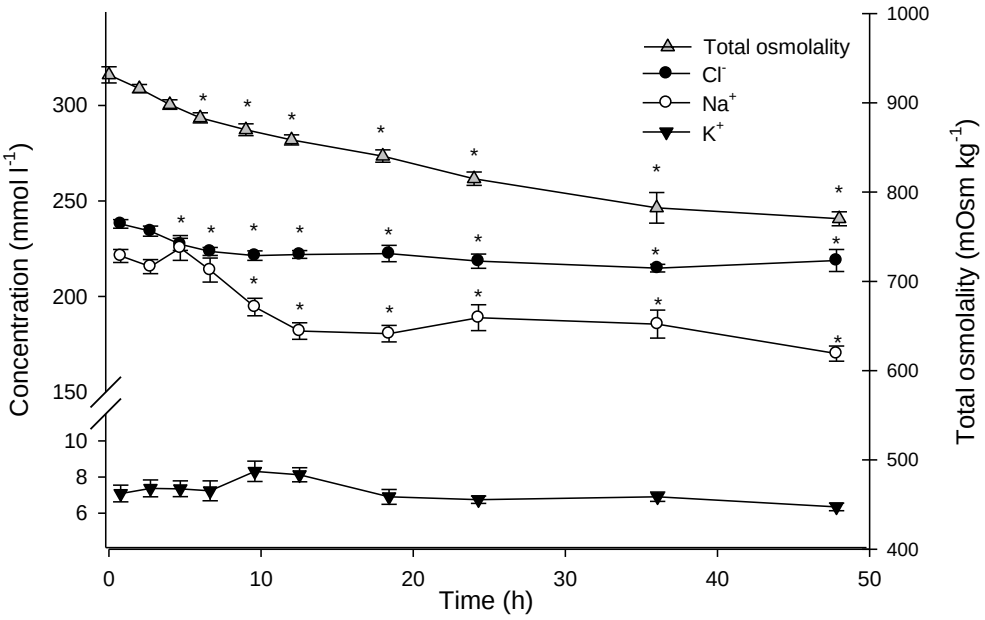

Figure 3.4. Total osmolality (right ordinate) and the concentrations of major plasma ions (left ordinate) in sharks exposed to 21‰ salinity. Values shown are means±SEM; n=6-9. Asterisks (*) indicate significant difference from control *t*=0 in 100% SW; $p<0.05$.

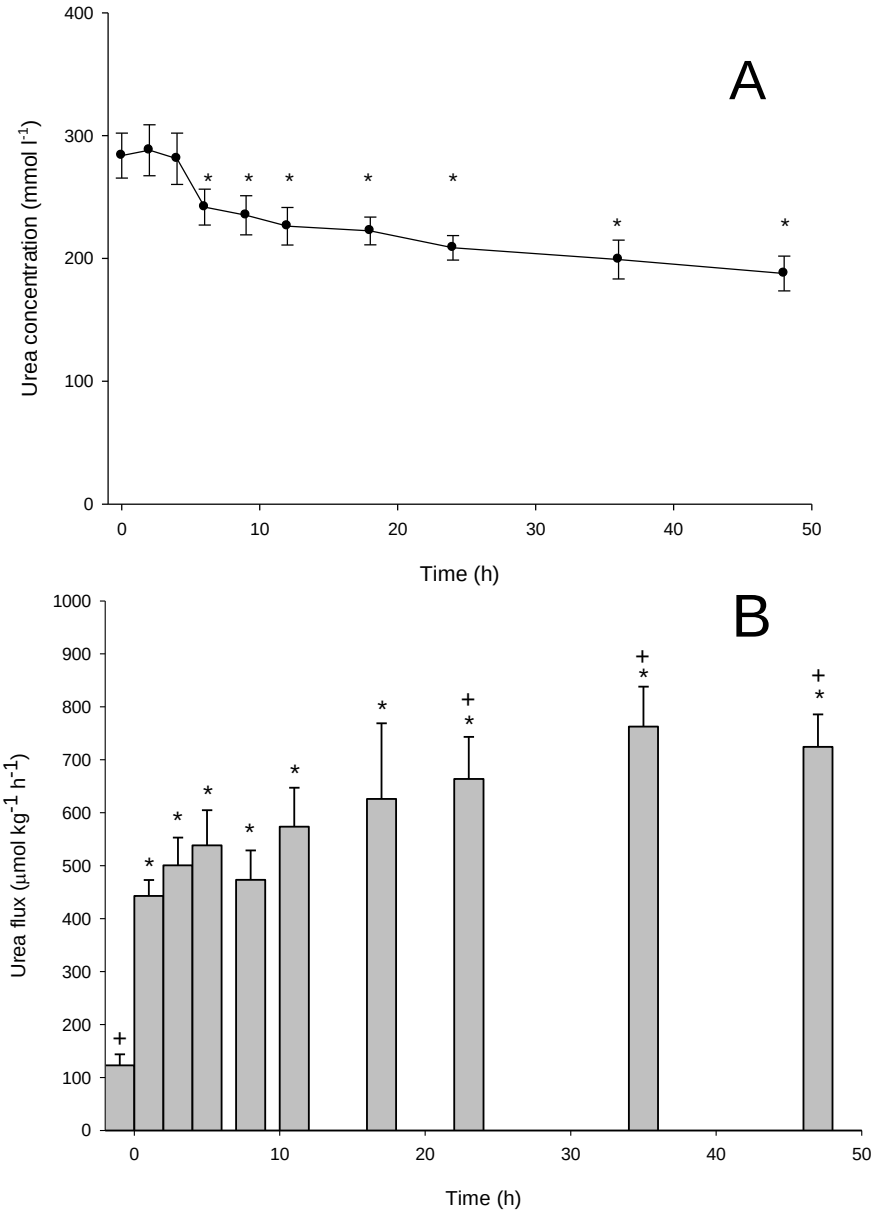

Figure 3.5. A) Concentration of urea in blood plasma of sharks exposed to 21‰ salinity. (n=7-9; means±SEM) Asterisks (*) indicate significant difference from control *t*=0 in 100% SW; p<0.05. **B) Rates of urea efflux from sharks measured by appearance of urea in water** (n=15-16; means±SEM) Asterisks (*) indicate significant difference from pre-exposure control period and plus signs (+) indicate significant difference from *t*=0-2h period; p<0.05.

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Chapter IV

Cloning and characterization of shark

Na^+/H^+ Exchanger isoforms NHE2 and NHE3 from *Squalus suckleyi*

Introduction

Several Na^+/H^+ exchangers, or NHEs (HUGO gene nomenclature: SLC9A subfamily) have been discovered in elasmobranchs and other fishes. Cells of the gills and kidneys of many fishes are known to possess at least one NHE, and several have been shown to possess both isoforms NHE2 and NHE3 (Choe et al. 2005; Claiborne et al. 2008; Hirata et al. 2003; Li et al. 2013). One of the remaining challenges for fish physiology is to delineate the specific functions of NHE isoforms in these specific tissues.

NHE proteins mediate the secondary active transport of one extracellular sodium ion for one intracellular proton. The activity of NHEs has been demonstrated to be involved in regulation of cell volume and intracellular pH (Boron and Boulpaep 1983;Grinstein et al. 1983) and transepithelial sodium transport (Knickelbein et al. 1983), and it has also been implicated in systemic pH balance and sodium uptake in fishes (Claiborne et al. 1997; Claiborne et al. 2002; Dymowska et al. 2012; Evans 1982; Krogh 1939).

Evidence for NHEs in fish gills comes from physiological, immunohistochemical, and molecular data. Many studies have shown evidence for Na^+/H^+ exchange on a macroscopic scale (Claiborne et al. 1994; Claiborne et al. 1997; Evans 1982; Krogh 1939). In fact, this mechanism seems to be responsible for almost all net influx of Na^+ and net efflux of acid (H^+ and/or NH_4^+) across the gills of marine fishes, including elasmobranchs, teleosts, and hagfishes (Bentley et al. 1976; Claiborne et al. 2002; Evans 1982; Evans 1984a; Evans and Piermarini 2005). Claiborne et al. (1999) used degenerate primers to

clone the first NHE from a fish (Long-horned sculpin *Myoxocephalus octodecimspinosus*) gill. Since then, homologues of human NHE2 and NHE3 have been cloned from the gills of several fish species including the Atlantic stingray *Dasyatis sabina,* Atlantic spiny dogfish *Squalus acanthias*, and Osorezan dace *Tribolodon hakonensis*, among others (Choe et al. 2005; Claiborne et al. 2008; Hirata et al. 2003). Interestingly, all of the NHEs cloned from fish gills have been homologues of either NHE2 or NHE3, and attempts to localize other NHE isoforms (*e.g.* NHE1) to gill cells have returned negative results (Yan et al. 2007). Concurrent with the cloning of the first gill NHE was also an era involving heavy use of immunochemistry in comparative physiology. Antibody reactivity indicating the presence of NHEs was observed in the gills of many species, and this is now considered a general phenomenon (Choe et al. 2007; Claiborne et al. 2008; Edwards et al. 1999; Edwards et al. 2002; Hirata et al. 2003;Wilson et al. 2000a,b). NHE2 and NHE3 are highly expressed in the ionocytes, or mitochondrion-rich cells, of the gill epithelium (Ballantyne and Robinson 2010; Choe et al. 2005; Choe et al. 2007; Claiborne et al. 2008). Interestingly, a subset of ionocytes has been observed to express both isoforms on the apical surface of the cells (Choe et al. 2007; Claiborne et al. 2008), leading to questions regarding the role of each isoform.

The most frequently discussed roles for NHEs are in systemic pH and sodium homeostasis (Choe et al. 2007; Donowitz et al. 2013; Evans and Piermarini 2005). Inferences into isoform-specific differences have been primarily based on changes in abundance of mRNA or immunoreactive protein.

Tresguerres et al. (2005) found that NHE2-like protein in the membrane-enriched fractions of gill homogenates from Pacific dogfish increased in response to systemic acidosis. In the euryhaline stingray, *Dasyatis sabina*, Choe et al. (2005) found NHE3 mRNA increased in brackish water versus seawater, and similar results were seen by Reilly et al. (2011) in the bull shark *Carcharhinus leucas*. In *D. sabina*, NHE2 mRNA did not change in response to brackish water while NHE3 mRNA increased, but during acidosis, NHE3 expression did not change (Choe et al. 2005). Experiments in non-elasmobranch fishes show similar results. NHE2-like protein also increased in gill membrane-enriched fractions from hagfish experiencing acidosis (Parks et al. 2007). In rainbow trout, NHE2 mRNA increased during acidosis, while NHE3 mRNA did not (Ivanis et al. 2008). These results are consistent with a role for NHE2 in acid excretion and for NHE3 in sodium absorption.

At the molecular level, the mammalian isoforms NHE1, NHE2, and NHE3 have been extensively studied (Brett et al. 2005; Donowitz et al. 2013; Franchi et al. 1986; Kapus et al. 1994; Lee et al. 2011). However, nearly all molecular analyses have exclusively investigated NHE in mammals. The best studied isoforms in terms of structure/function relationships are NHE1 and NHE3. These proteins consist of an approximately 500 amino acid amino-terminal transmembrane domain and an approximately 300 amino acid carboxyl-terminal regulatory domain (Donowitz et al. 2009; Lee et al. 2011; Wakabayashi et al. 1997; Wakabayashi et al. 2000). The protein is predicted to have 12 membrane-spanning alpha helices (Wakabayashi et al. 2000), although a topology model

based on homology to the bacterial Nha transporter assigns these domains differently (Landau et al. 2007; Lee et al. 2011). Some membrane spanning segments, including the putative fourth transmembrane domain (TM IV) of the Wakabayashi model, contain partially disordered regions rather than assuming a classical alpha helical structure (Lee et al. 2011; Slepkov et al. 2005). TM IV is also known to be involved in sodium binding, transport, and the action of amiloride-based drugs (Slepkov et al. 2005). Other sections, including TM IX are known to be important but are not as well conserved across NHE isoforms (Slepkov et al. 2007). The other NHE isoforms are predicted to have similar overall structures, especially in the greater transmembrane domain (Borgese et al. 1992; Brett et al. 2005; Tzeng et al. 2011).

Nine paralogs of NHE have been identified in the human genome, and eight have been identified in the genome of the chondricthyan elephant shark, *Callorhinchus milii* (Donowitz et al. 2013; Venkatesh et al. 2007). Of these, only NHE2 and NHE3 have been cloned from fish gills. In mammalian epithelial tissues including the collecting duct of the kidney cortex, both NHE2 and NHE3 (and NHE1) are located in the cell membrane, with NHE3 frequently moving between the cell membrane and recycling endosomes while NHE2 mostly resides in the cell membrane after initial insertion (Brett et al. 2005). It has been shown, however, that there is also an intracellular pool of NHE2 that awaits recruitment to the cell membrane in response to low intracellular pH (pH_i) (Gens et al. 2007).

In addition to the molecular analysis, functional assays of NHEs have been almost entirely limited to mammalian species. Many experiments on mammalian NHEs1-3 cloned from several species and expressed in several systems have revealed some general patterns regarding the functional aspects of these isoforms (Borgese et al. 1992; Franchi et al. 1986; Kapus et al. 1994; Orlowski 1993). For reviews, see Alexander and Grinstein (2009), Brett et al. (2005), Donowitz and Li (2007), Donowitz et al. (2009, 2013), and Goss and Grinstein (1996). In general, mammalian NHE2 is susceptible to inhibition by amiloride and amiloride analogues such as EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride), while NHE3 is around 10-50 times more resistant (Masereel et al. 2003). The few non-mammalian NHEs to be cloned, expressed and functionally analyzed include a mosquito NHE3 (Pullikuth et al. 2006) and an NHE3 from the freshwater dace *Tribolodon hakonensis*, which was found to be slightly more resistant to EIPA than is human NHE3 (Hirata et al. 2003). The other NHEs cloned from fishes and other organisms have not been functionally analyzed, yet physiological studies proceed on the assumption that all NHEs function similarly and have similar inhibition profiles to mammalian NHEs.

The goals of this study were to clone NHE2 and NHE3 from the same elasmobranch fish species, the North Pacific spiny dogfish (dfNHE2, dfNHE3), to express them in active form in a convenient NHE-deficient cell line, and to examine their functionality and susceptibility to inhibition by common sodium transport inhibiting drugs. In particular, I have examined the effects of amiloride (a classical NHE inhibitor), phenamil (a putative sodium channel inhibitor that is

assumed to not affect NHEs) and EIPA (supposedly a more potent and specific antagonist of NHEs) for their effects on dfNHE2 and dfNHE3 (Kleyman and Cragoe 1988). Furthermore, the potential to discriminate the activities of dfNHE2 and dfNHE3 through proper dosing of EIPA was evaluated.

Materials and methods

Animals

North Pacific spiny dogfish sharks (*Squalus suckleyi*) were caught by hook and line from the Trevor Channel (Vancouver Island, BC, Canada) and immediately transferred to the Bamfield Marine Sciences Centre, where they were held in a tank provided with flowing seawater. Fish were fed, fasted for four days, and euthanized with an overdose of MS-222. Tissues were dissected, snap frozen in liquid nitrogen, and held at -80 °C. All experimental protocols were approved by the University of Alberta and Bamfield Marine Sciences Centre animal care committees.

RNA isolation and cloning of full cDNA

RNA was isolated from freshly thawed tissues using TRIzol Reagent (Life Technologies, Carlsbad, California), treated with DNAse I and purified using RNeasy Mini spin columns (Qiagen Canada, Montreal, Quebec) according to the manufacturer's instructions. Purity was checked by measuring absorbance at 230, 260, and 280 nm on a spectrophotometer and integrity was evaluated using denaturing agarose gel electrophoresis. RNA was converted to cDNA through the Fermentas reverse transcriptase reaction (Thermo Fisher Scientific, Waltham, Massachusetts) using oligo-dT and random hexamer primers.

Several partial sequences of NHE-like transcripts were discovered in a dogfish gill transcriptome profile database, and a PCR-based strategy was used to amplify overlapping fragments and deduce the full coding sequences (CDS) of dogfish NHE2 and dogfish NHE3 cDNA (referred to as dfNHE2 and dfNHE3,

respectively). Several rounds of 5' and 3' Rapid Amplification of cDNA Ends (RACE) were employed. Takara 5' and 3' RACE and SMARTer 5' and 3' RACE reactions (Clontech Laboratories, Mountain View, California) were alternately employed to amplify several overlapping sequences to span the full CDS up to the 5' and 3' UTRs. Finally, two full-length transcripts were amplified using the following primers: 5'-TTAAATACCTGTGACCATGGGCGGTG-3' and 5'-CGCTTTCATAATGTTGACCGAGATTACCAA-3' for dfNHE2 and 5'-GCCACGATGGGGAGAGATAGGAGCGAGTGTGC-3' and 5'-GGACTTGGGATTGACTTAGAGTTACATTGATG-3' for dfNHE3. The products were then cloned into the pTargeT mammalian expression vector (Promega, Madison, Wisconsin). The constructs were sequenced and the full insert sequences were identical to the deduced CDS. The entire nucleotide sequence of each cDNA was sequenced to a minimum of 4X sequencing replication using cDNA from two individual sharks.

Sequence analysis and phylogenetics

A BLASTp search of the NCBI nonredundant protein database revealed high sequence similarity to other NHE2 (slc9a2) and NHE3 (slc9a3) sequences. Selected NHE amino acid sequences were aligned using CLUSTAL Omega and curated using GBLOCKS with less stringent parameters. Maximum likelihood phylogenetic analysis was conducted using PhyML through the program SEAVIEW using the LG substitution model and an automatically optimized gamma distribution of across-site rate variation. Neighbor-joining methods and maximum parsimony methods also produced optimal tree topologies identical to

that produced by the maximum likelihood method. Reliability of separations in the maximum likelihood tree was assessed through 500 bootstrap replicates.

Amino acid sequences of each dfNHE isoform were aligned with select related sequences using CLUSTAL Omega. Canonical phosphorylation sites for protein kinase A (PKA) and serum/glucocorticoid regulated kinase (SGK) were inferred using the GPS 2.1 algorithm with a high threshold value (http://gps.biocuckoo.org).

Expression in antiporter-deficient cells

The pTargeT/dfNHE2 and pTargeT/dfNHE3 constructs were designed for expression in the AP-1 cell line. This line is a derivative of the Chinese hamster ovary cell line that is completely deficient in NHE activity (Rotin et al. 1989). The cells were grown in plastic tissue culture dishes at 37° C in a humidified atmosphere of 5% CO_2/95% air and in a medium of MEM-alpha + 10% fetal bovine serum + 200 units penicillin/streptomycin per ml + 25 mM HEPES, pH=7.4. The plasmid constructs were transfected into AP-1 cells using Lipofectamine-2000 reagent (Life Technologies, Burlington, Ontario). Subsequently the growth medium was supplemented with 400 μg/ml geneticin (G418). After resistant colonies developed, secondary selection by acid loading was performed as described by Wang et al. (1998). Cultures were maintained by regular re-establishment from frozen stocks, and cultures were used for experiments within 6-12 passages after transfection.

Na^+/H^+ exchange assay

NHE activity was calculated from the initial rate of Na^+-induced recovery of intracellular pH (pH_i) after induced cellular acidification as described previously (Murtazina et al. 2001). Cells were grown to 60-100% confluence on glass coverslips, and pH_i was monitored fluorimetrically using BCECF (2-,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; Molecular Probes). The coverslip of BCECF-loaded cells was placed in normal buffer containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH=7.4 in a cuvette holder at 37° C with constant stirring. 30 mM ammonium chloride was added for three minutes before the coverslip was transferred to sodium-free buffer in which the NaCl component of normal buffer was replaced by *N*-methyl-D-glucamine HCl. After induction of acidification for 30 seconds, pH_i recovery was measured in normal buffer. The initial rate of pH_i recovery was calculated from the first 20 seconds of recovery. Calibration of BCECF fluorescence to intracellular pH was conducted by the high K^+/nigericin method (Chaillet and Boron 1985; Silva et al. 1997). For inhibitor tests, the effect of each treatment was assessed through a double pulse protocol. One cycle of acidification and recovery was measured in the absence of drug. Then another cycle of acidification and recovery was measured with the drug present in both the sodium-free and normal buffers. The measurements were highly repeatable, with activity in the second pulse averaging 100±5% of the first pulse. Application of 0.1% DMSO as a vehicle control caused no significant effect. All drugs and materials were purchased from Sigma-Aldrich (Oakville, Ontario) unless noted otherwise.

Data presentation and statistical analysis.

Activity data are shown as means ± SEM normalized to activity in the absence of drug. Values at zero drug concentration represent the vehicle control treatment. Data for inhibition profiles were fitted to sigmoidal dose-response curves with Hill coefficients using SigmaPlot v11.0.

Results

Using PCR and RACE, the full CDS of the *S. suckleyi* dogfish NHE2 and dogfish NHE3 (dfNHE2 and dfNHE3, respectively) were deduced and cloned from gill cDNA. The *S. suckleyi* dfNHE2 cDNA contains a 2310 bp ORF encoding a 769 amino acid protein, followed by a 150 bp 3' UTR including the poly-A tail and preceded by a 5' UTR of at least 225 bp. The *S. suckleyi* dfNHE3 cDNA contains a 2505 bp ORF encoding an 834 amino acid protein, an 887 bp 3' UTR with poly-A tail and a 5' UTR of at least 84 bp.

Maximum likelihood phylogenetic inference supports the identification of the cloned sequences as members of the NHE2 (slc9a2) and NHE3 (slc9a3) clades (Fig. 4.1). The phylogenetic tree obtained here is consistent with the diversification of the NHE (slc9a) family early in the ancestry of the vertebrates.

The deduced sequence of the *S. suckleyi* dfNHE2 differs from the closely related *S. acanthias* NHE2 by seven or eight amino acid residues (Fig. 4.2). (One residue was unresolved in the published *S. acanthias* NHE2.) The fourth transmembrane domain (TM IV) is almost perfectly conserved across all of the examined NHE2s. Two residues in *S. suckleyi* dfNHE2, S602 and S625, are predicted to be SGK (serum- and glucocorticoid-regulated kinase) phosphorylation sites.

The deduced sequence of the *S. suckleyi* dfNHE3 was highly similar to other vertebrate NHE3s (Fig. 4.3). The dfNHE3 sequence shares 87% amino acid identity with the NHE3 from the gill of the Japanese houndshark, *Triakis scyllium*. A PKA (protein kinase A) phosphorylation site, S569 in dfNHE3, is

conserved across all NHE3s examined. Position S619 in dfNHE3 is predicted to be either an SGK or PKA phosphorylation site. The sequence of TM IV is strongly conserved across vertebrate NHEs, but dfNHE3 differs from the human and *T. scyllium* NHE3 sequence at two positions, N127 and I138.

When expressed in Na^+/H^+ exchange-deficient AP-1 cells, dfNHE2 and dfNHE3 each rescue Na^+/H^+ exchange activity and allow recovery and survival after an induced acid load. Both untransfected AP-1 cells and cells transfected with the empty vector showed no Na^+-induced recovery and did not survive after acid loading. An illustrative example of Na^+-induced recovery after acid loading in AP-1/dfNHE2 cells is shown in Figure 4.4. Upon exposure to NH_4Cl, the pH_i quickly increases due to NH_3 diffusion across the membrane and gradually decreases as NH_4^+ enters the cell. A sodium- and ammonia-free medium next causes exit of NH_3 and deprotonation of intracellular NH_4^+; the NH_3 freely diffuses out of the cell while protons remain inside, causing an acidification of pH_i. If Na^+ is then supplied, the expressed dfNHE2 or dfNHE3 functions to restore pH_i nearly to the initial value in less than two minutes.

The sensitivity of expressed dfNHE2 and dfNHE3 to some common sodium transport inhibitors was assessed at 135 mmol Na^+ l^{-1}. Figure 4.5 shows the dose-response curves for inhibition of dfNHE2 by amiloride and EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride). The inhibitor concentration sufficient for 50% inhibition (IC_{50}) of dfNHE2 was estimated at 55 μmol l^{-1} for amiloride and 4.5 μmol l^{-1} for EIPA. Exposure to 100 μmol l^{-1} phenamil inhibited dfNHE2 activity by less than 10%. As shown in Figure 4.6, dfNHE3 exhibited different

sensitivities, with an estimated IC_{50} of 24 μmol l^{-1} for amiloride and 9 μmol l^{-1} for EIPA. Phenamil at 100 μmol l^{-1} inhibited dfNHE3 activity by only 16±6%.

Discussion

Comparison of the *S. suckleyi* dfNHE2 with the previously reported *S. acanthias* NHE2 reveals either seven or eight amino acid substitutions, as one residue was unresolved in the published *S. acanthias* NHE2 sequence (Claiborne et al. 2008). This evidence corroborates the genetic and life history traits that have been used by Verissimo et al. (2010) and Ebert et al. (2010) to distinguish these two sharks as distinct species. All but one or two of the amino acid substitutions occur very near the carboxyl-terminal tail of the protein, which is not known to have any particular function and may be somewhat free to vary. That said, there have been no structural studies of NHE2 and little is known about the function of various domains except what is inferred based on other NHE isoforms (Donowitz et al. 2013).

Two residues in *S. suckleyi* dfNHE2, S602 and S625, are predicted to be SGK (serum- and glucocorticoid-regulated kinase) phosphorylation sites. SGK has been cloned from *S. acanthias*, and transcription can be induced by hypertonic medium (Waldegger et al. 1998). Work by Kapus et al. (1994) showed that rat NHE2 was stimulated by hypertonic medium, and one study on human NHE2 reported stimulation by serum, which may have influenced SGK (McSwine et al. 1998). It is therefore possible that similar mechanisms may act on dfNHE2. Other reports of acute regulation of NHE2 include one report of recruitment of recombinant human NHE2-CFP to the cell membrane within three minutes of cellular acidification (Gens et al. 2007). It was shown in the same study that the pH-sensitive element lay somewhere in the greater transmembrane domain

(amino acids 1-551). Also, it has been reported that rat NHE2 is stimulated by cAMP, PKA, and PKC but unaffected by cGMP (Kandasamy et al. 1995); however, cAMP, PKA, and PKC were reported to inhibit human NHE2 expressed in Caco-2 BBE cells (McSwine et al. 1998). Whether these pathways regulate dfNHE2 in similar ways to mammalian NHE2 is unknown but could be tested using the expression system I have established.

The dfNHE3 cloned in the present study is very similar to other vertebrate NHE3s, including the recently reported *Triakis scyllium* gill NHE3, the first shark NHE3 to be cloned and reported (Li et al. 2013). A protein kinase A phosphorylation site, S569 in dfNHE3, is conserved across all NHE3s examined. The homologous residue in rat NHE3 is one of at least three serines involved in cAMP- and PKA-mediated inhibition (Donowitz and Li 2007). Another interesting region precedes S619 in dfNHE3. In rat NHE3, this serine residue is known to be the most important PKA phosphorylation site involved in acute downregulation in response to cAMP (Kurashima et al. 1997). In dfNHE3, however, S619 is preceded by the sequence ENRVKS619, which is predicted by the GPS 2.1 algorithm to be slightly more likely to be an SGK site than a PKA site. In mammalian NHE3s, SGK stimulates NHE3 activity through phosphorylation at a different site (Wang et al. 2005), as well as by phosphorylation-independent mechanisms (Donowitz and Li 2007).

The sequence of TM IV is strongly conserved across vertebrate NHEs and is known to be important in Na^+ affinity and in the action of amiloride-based inhibitor drugs (Lee et al. 2011; Slepkov et al. 2005; Tzeng et al. 2011). Table 4.1

shows the sequences of TM IV from human NHE1-3 aligned with dfNHE2 and dfNHE3. The sequence of dfNHE2 is nearly identical to human NHE2 (also see Figure 4.2), but dfNHE3 differs from the human, *T. scyllium,* and *Petromyzon marinus* NHE3 sequence at two positions, N127 and I138 in dfNHE3 (also see Figure 4.3). Regarding the consensus sequence as ancestral, the V138I substitution is fairly conservative, while the T127N substitution is nonconservative. Though the role of T127 within TM IV is not known, substitution of this position in NHE1 with cysteine greatly reduces transport activity (Slepkov et al. 2005).

The activity of dfNHE2 and dfNHE3 were sensitive to amiloride and EIPA but resistant to phenamil. dfNHE2 is considerably more resistant to amiloride and EIPA than is human NHE2, while dfNHE3 is considerably less resistant than human NHE3. Because these differences are present even though the few residues known to be important are identical between the two species, other residues must also be important in Na^+ affinity, amiloride binding, or both. For example, in dfNHE3, substitution T127N may change the shape of the Na^+ and/or amiloride-binding pocket(s), potentially changing the transporter's substrate affinity and sensitivity to inhibitors (Lee et al. 2011; Touret et al. 2001). In addition, mutations in putative TM IX of NHE1 have been shown to affect sensitivity to amiloride, but this region is not well conserved across NHE isoforms (Khadilkar et al. 2001; Slepkov et al. 2007).

This is the first examination of inhibitor sensitivity in any non-mammalian NHE2 and NHE3. The results will be useful for studies of systemic pH and

sodium homeostasis as well as in mechanistic studies of NHE activity at the cellular and subcellular levels. In any applications of sodium transport inhibitors to fishes, these data should be considered rather than relying on data from mammalian systems. Future experiments done in a physiological saline or other solution of 135 mmol l^{-1} Na^+ may use 100 μmol l^{-1} amiloride to inhibit NHEs, though NHE2 may still exhibit around 20% activity. As little as 30 μmol l^{-1} EIPA should provide maximal inhibition of NHEs. That said, additional trials around the estimated IC_{50} values for dfNHE2 are underway to reduce uncertainty in the estimate. The action of 100 μmol l^{-1} phenamil on NHEs is negligible, and it can therefore be used to block sodium channels without affecting NHE2 or NHE3. Unfortunately, NHE2 and NHE3 cannot be distinguished through the use of amiloride and EIPA because sensitivities of these isoforms are too similar.

In solutions of greater or lesser Na^+ concentration, the concentration of inhibitor should be adjusted, owing to the competitive mode of inhibition exhibited by these drugs (Kleyman and Cragoe 1988; Masereel et al. 2003). While K_i values have not been determined in this study, they can be estimated using the relationship $IC_{50}=K_i (1 + S/K_m)$, where S is the concentration of Na^+ and K_m is the apparent affinity for Na^+ (Cheng and Prusoff 1973). Future measurement of apparent K_m values for these NHEs will allow calculation of K_i values and appropriate inhibitor concentrations under any concentration of Na^+ without resorting to assumptions based on mammalian affinity values.

Previous experiments involving the application of these drugs to non-mammalian organisms can now be revisited. Many studies, especially those

conducted in freshwater, have used inhibitor concentrations that seem appropriate in light of the data reported here; however, the present study should be repeated using NHEs from several diverse fish taxa. In one freshwater cyprinid species, *Tribolodon hakonensis*, an NHE3 was cloned, expressed, and tested for EIPA sensitivity in a similar manner (Hirata et al. 2003). That study reported an IC_{50} in 1 mmol Na^+ l^{-1} of around 10 μmol EIPA l^{-1}. Accounting for the Na^+ concentration of the assay medium, this implies that the *T. hakonensis* NHE3 exhibits either a much greater affinity for Na^+ or a much lower affinity for EIPA than either dfNHE3 or human NHE3, or some combination of these factors. A cloned mosquito NHE3 was rather prematurely labeled unaffected by amiloride and EIPA due to insufficient data collection (Pullikuth et al. 2006). To the best of my knowledge, there has been no other report of inhibitor sensitivity for any other cloned NHE from any non-mammalian organism.

Conclusions

The dfNHE2 and dfNHE3 have been cloned and sequenced from the shark *Squalus suckleyi*. These are the first NHE2 and NHE3 to be cloned from the same non-mammalian organism. Sensitivity to amiloride and EIPA and resistance to phenamil were determined for each isoform stably expressed in AP-1 cells, and dfNHE2 was found to be considerable more resistant to these drugs than is human NHE2, while dfNHE3 is more sensitive to these drugs than is human NHE3. These data will be useful not only for general studies of sodium transport in fishes, but especially so in future attempts to discriminate the roles of these two isoforms in systemic pH and sodium homeostasis.

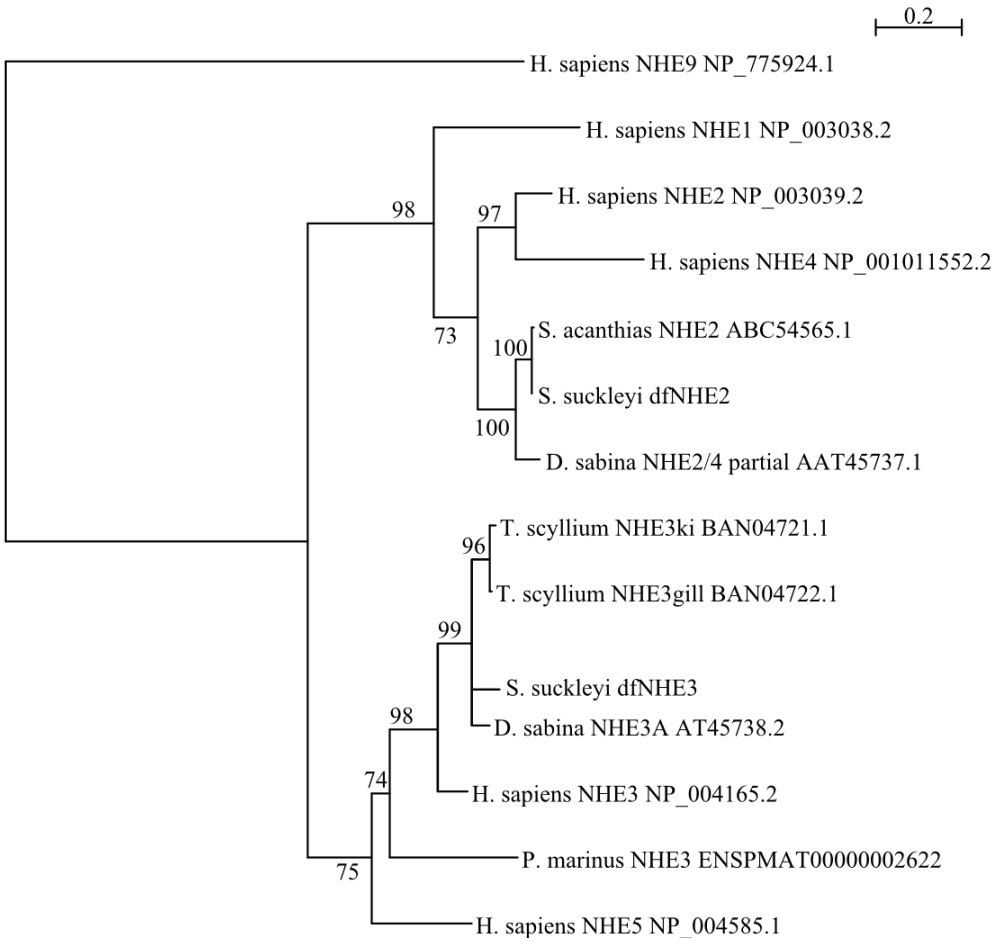

Figure 4.1. Maximum likelihood (PhyML) phylogenetic tree of the cloned *S. suckleyi* dfNHE2 and dfNHE3 among other vertebrate NHEs. Numbers indicate the percentage of 500 bootstrap replicates that supported the separation. Branch length represents degree of divergence, with the scale bar indicating the distance representing 0.2 substitutions per position. Divisions with less than 50% bootstrap support have been collapsed. Genbank identification numbers are given at the end of the protein name, except for the *P. marinus* protein, which was retrieved from Ensembl. List of genera: H., *Homo*, S., *Squalus*, D., *Dasyatis*, T., *Triakis*, P., *Petromyzon*.

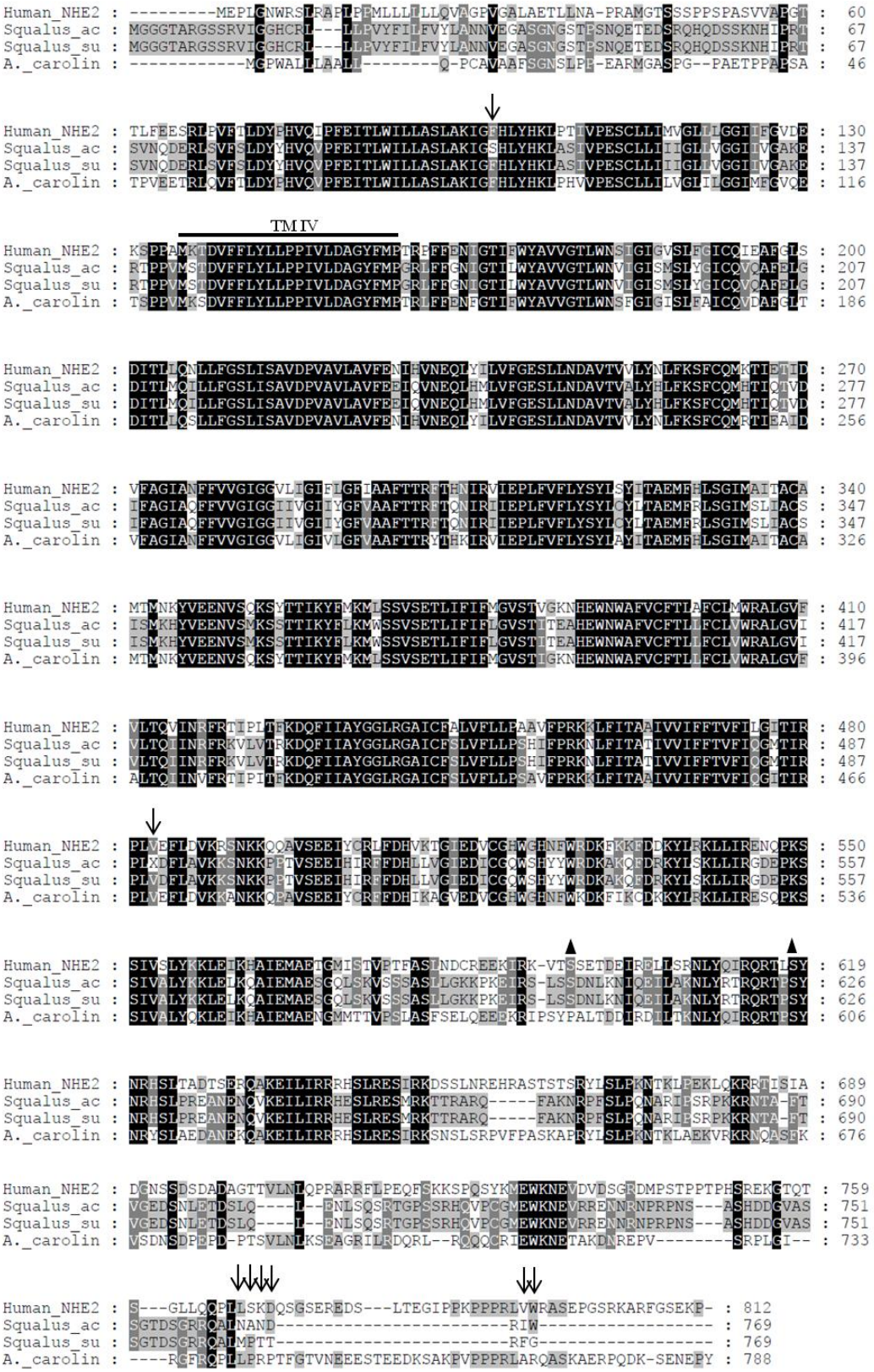
TM IV

Figure 4.2. (previous page) **CLUSTAL Omega alignment of deduced amino acid sequences of *S. suckeyi* dfNHE2 with *S. acanthias* NHE2 and related sequences.** Positions highlighted in black are strongly conserved and positions in grey are moderately conserved. Arrows indicate differences between congeneric dogfish sequences. The fourth transmembrane domain, which is critical for transport function, is indicated by an overline. Triangles indicate likely SGK phosphorylation sites in *S. suckleyi* dfNHE2 as predicted by the GPS 2.1 algorithm. Sequence labels: Human NHE2, NP_003039.2; Squalus_ac, *Squalus acanthias* NHE2 ABC54565.1; Squalus_su, *Squalus suckleyi* dfNHE2; A._carolin, *Anolis carolinensis* NHE2-like XP_003224334.1.

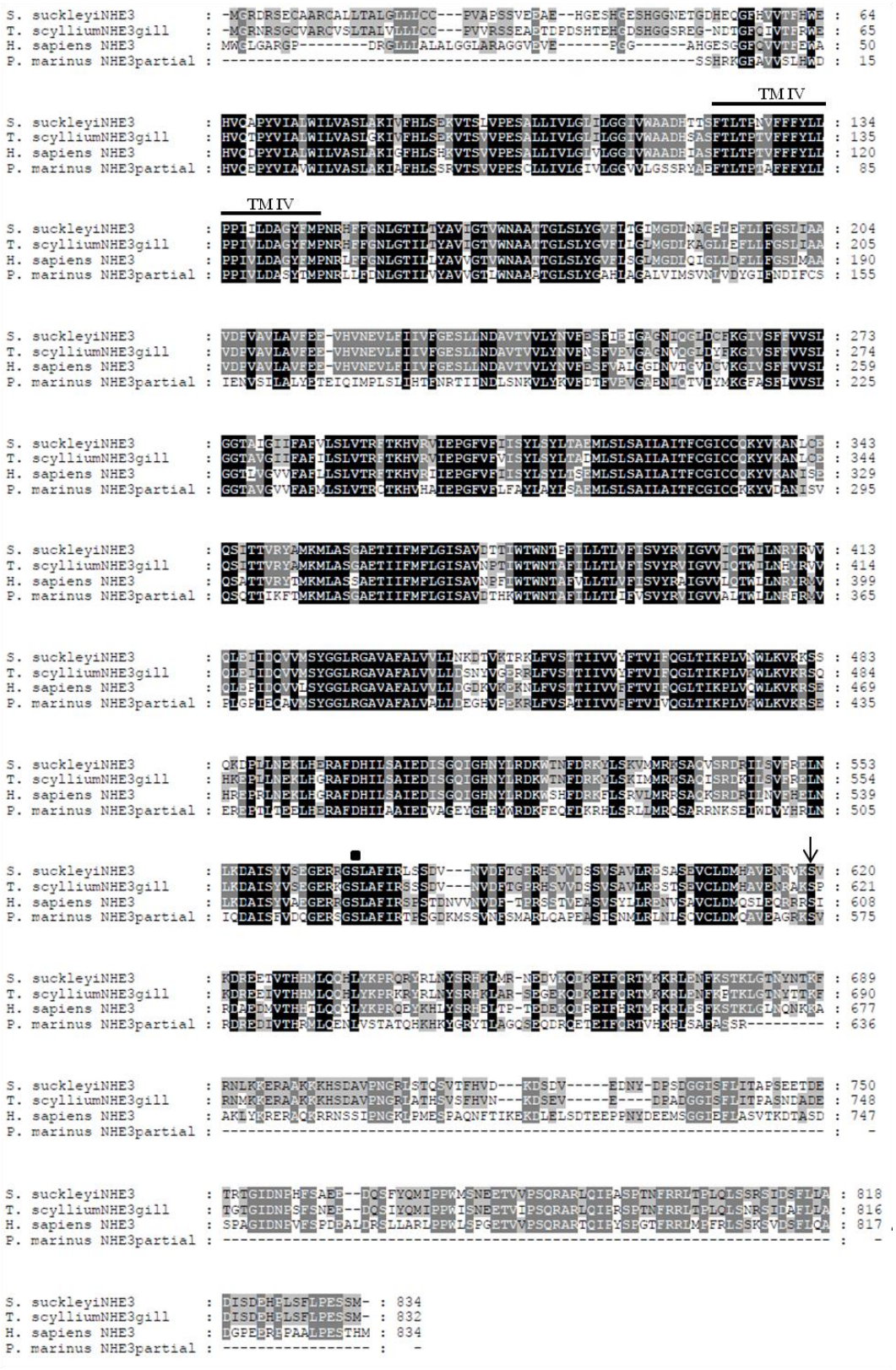
TM IV
TM IV

Figure 4.3. (previous page) **CLUSTAL Omega alignment of deduced amino acid sequences of *S. suckleyi* dfNHE3 and related sequences.** Positions highlighted in black are strongly conserved and positions in grey are moderately conserved. The fourth transmembrane domain, which is critical for transport function, is indicated by an overline. The black square indicates a PKA phosphorylation site that is conserved across all of these NHE3s. An arrow indicates an interesting position, S619 in *S. suckleyi* dfNHE3 that is predicted to be either an SGK or PKA phosphorylation site; the position is an important inhibitory PKA site in rat NHE3. *T. scyllium* NHE3gill BAN04722.1, *H. sapiens* NHE3 NP_004165.2, *P. marinus* NHE3partial ENSPMAT00000002622.

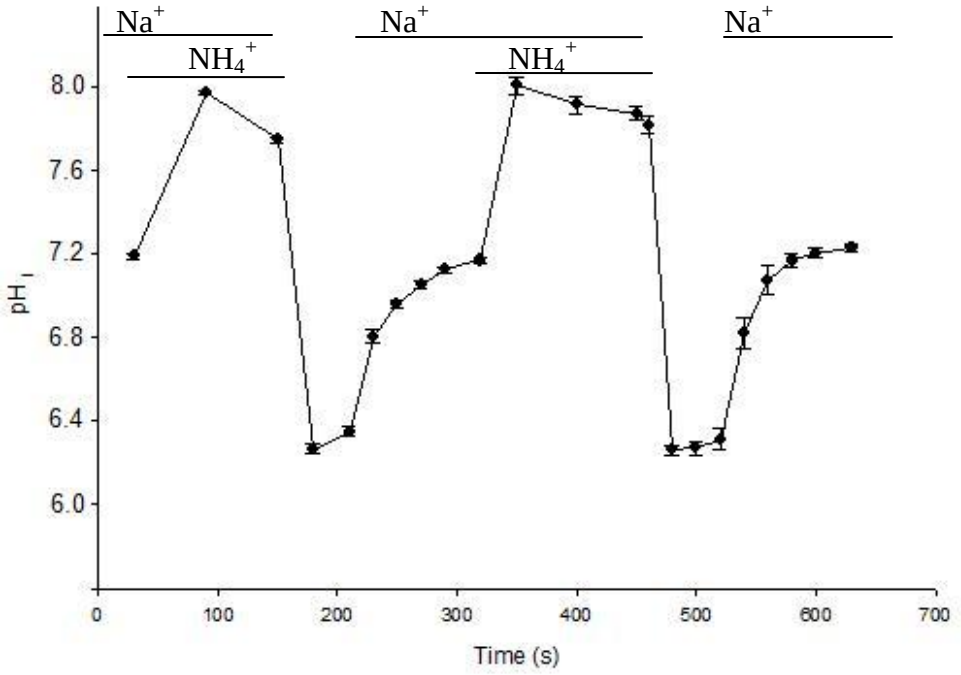

Figure 4.4. Illustrative trace of the NHE activity assay. Cells loaded with BCECF were exposed to 30 mmol l^{-1} NH_4Cl in normal buffer for three minutes and then placed in Na^+-free buffer to induce cellular acidification. The cells were then returned to normal Na^+-containing buffer and the rate of pH_i recovery was monitored. The process was then repeated with inhibitors in the Na^+-free and normal buffers. NHE activity was calculated from the slope of the first 20 seconds of Na^+-induced recovery. Time periods during which cells were exposed to Na^+ and to NH_4^+ are indicated. Actual measurements are much more frequent than in this illustrative trace. Values represent means ± SEM, n=8.

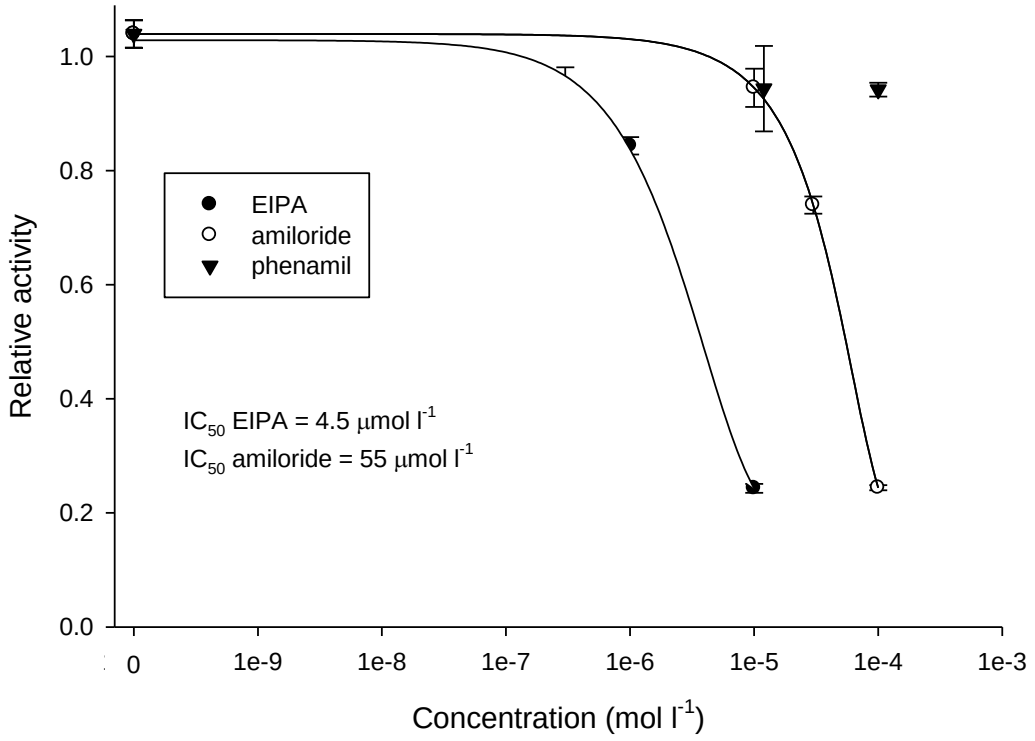

Figure 4.5. Dose-response curves showing the effects of select inhibitors on dfNHE2 activity. Values are mean ± SEM, n=4. The sigmoidal dose-response curves with Hill coefficients were fitted with SigmaPlot v11.0. Phenamil at 100 μmol l^{-1} caused less than 10% inhibition.

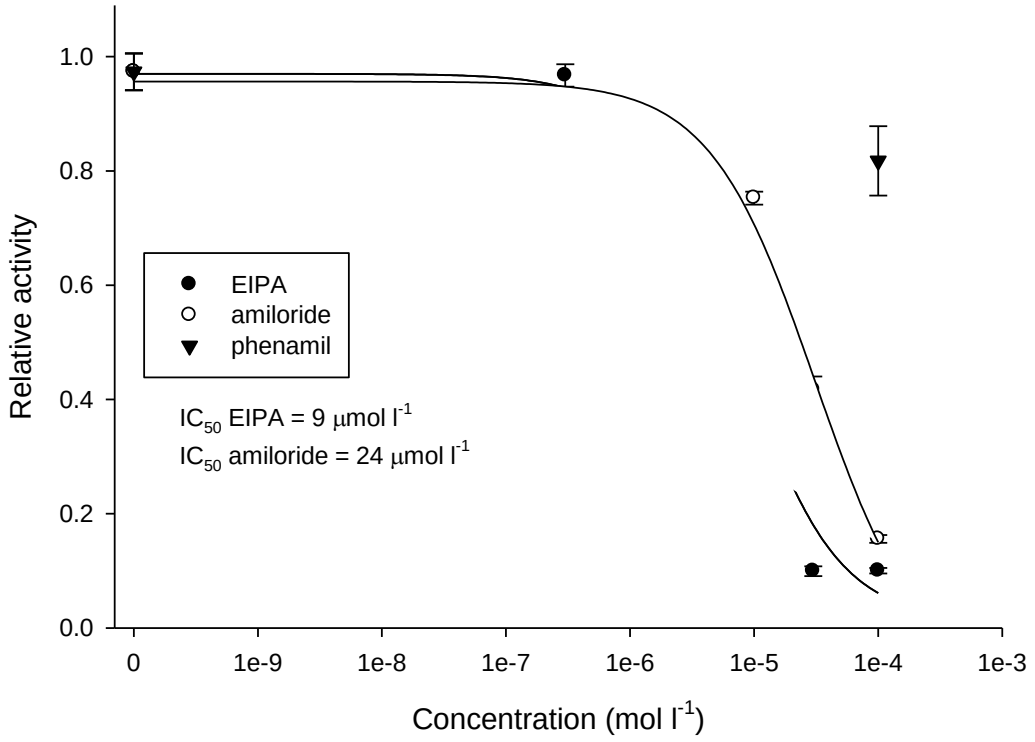

Figure 4.6. Dose-response curves showing the effects of select inhibitors on dfNHE3 activity. Values are mean ± SEM, n=4. The sigmoidal dose-response curves with Hill coefficients were fitted with SigmaPlot v11.0. Phenamil at 100 μmol l^{-1} caused 16±6% inhibition.

Table 4.1: **Alignment of TM IV sequences from several NHEs and corresponding IC_{50} values at 135 mmol l^{-1} Na^+.** TM IV is highly conserved and the underlined residues are known to be involved in Na^+ affinity and the action of amiloride-based drugs (Slepkov et al. 2005). dfNHE2 is considerably more resistant to amiloride and EIPA than human NHE2, while dfNHE3 is considerably less resistant than human NHE3. Because these differences are present even though the few residues known to be important are identical between the two species, other residues must also be important in Na^+ affinity, amiloride binding, or both. IC_{50} values for dfNHEs at 135 mmol l^{-1} Na^+ are from Figures 4.5 and 4.6. IC_{50} values for human NHEs are calculated at 135 mmol l^{-1} Na^+ from K_i values presented in Masereel et al. (2003) using the relationship $IC_{50}=K_i\ (1 + S/K_m)$, where S is the concentration of Na^+ and K_m is the apparent affinity for Na^+.

Protein	**Sequence**	**IC_{50} for amiloride (µmol l^{-1})**	**IC_{50} for EIPA (µmol l^{-1})**
Human NHE1	PPFLQSDV<u>FFL</u>FLLPPIILDA<u>G</u>YFL	23	0.3
Human NHE2	PPAMKTDV<u>FFL</u>YLLPPIVLDA<u>G</u>YFM	4	1.8
dfNHE2	PPVMSTDV<u>FFL</u>YLLPPIVLDA<u>G</u>YFM	55	4.5
Human NHE3	SFTLTPTV<u>FFF</u>YLLPPIVLDA<u>G</u>YFM	>100	67
dfNHE3	SFTLTPNV<u>FFF</u>YLLPPIILDA<u>G</u>YFM	24	9

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Chapter V

General discussion

Introduction

The overall goals of this thesis were to investigate some mechanisms of solute homeostasis in fishes, and in particular to incorporate the techniques of molecular physiology in these investigations. I have accomplished these overall goals as well as the specific objectives set out in Chapter I.

Briefly, the specific objectives restated were to:

1) examine the possibility of phosphate uptake across hagfish skin and gills,

2) measure uptake kinetics and investigate the mechanism of transport,

3) clone and sequence the phosphate transporter from the hagfish gill,

4) examine the ability of dogfish to acclimate to reduced salinity,

5) detail the time course of changes to solute homeostasis in reduced salinity,

6) clone and sequence the full-length dogfish gill NHE2 and NHE3,

7) express these NHEs in active form in stable cell lines, and

8) determine effective concentrations of NHE inhibitors for each isoform.

This General Discussion will first briefly summarize each of the data chapters. A discussion of the cellular and molecular physiology of NHE function in fishes is then offered, followed by some promising directions for future study. The techniques of molecular physiology hold great promise for complementing classical methods of fish physiology, and I hope that these studies and others like them will add to the new molecular dimension of comparative physiology.

Hagfish

In the study of phosphate uptake by the hagfish, I observed the absorption of inorganic phosphate into the gills, skin, and gut. This remarkable ability could provide the feeding hagfish with access to nutrients even before digestion and absorption via the gut are complete. This study was the first measurement of uptake of this inorganic nutrient across the extra-intestinal epithelia of any animal. Interestingly, after scaling up the measured uptake rates to the level of the whole animal, I found that the gills and skin could contribute significantly to whole-animal P balance. In isolated organ bath experiments, two mechanisms of uptake were observed; one saturable mechanism active at low phosphate concentrations, and one non-saturable mechanism that was dominant at higher concentrations. Interestingly, uptake rates in the gill and skin at 2 mmol phosphate l^{-1} were only slightly reduced under Na^+-free conditions or under exposure to the NaPi-II inhibitor phosphonoformic acid perhaps suggesting a Na^+ independent mode of transport. I suggest that the hagfish NaPi-II-like transporter that I have cloned may be one of several mechanisms at work in the gill and skin. The NaPi-II-like cDNA cloned from the hagfish gill is now available for future experiments including heterologous expression studies (*e.g. Xenopus* oocyte expression), localization, and characterization in hagfish tissues (see Future directions below).

At this time, the data concerning the direct involvement of the hagfish NaPi-II-like transporter in the observed phosphate uptake are equivocal. Most likely, the phosphate concentration (2 mmol l^{-1}) during the Na^+-free and phosphonoformic acid experiments was too high. The non-saturable pathway may

have overshadowed the saturable NaPi-II-like pathway and diminished the effect of Na^+ and PFA inhibition. In addition, the nominally Na^+-free experiments may have been impacted by carryover of small amounts of Na^+ on surface fluids or even by Na^+ leak out of the tissues. Given the relatively high affinity of this transporter, a small Na^+-linked uptake may still have been present. These tests should be repeated at a lower concentration of phosphate, and future control experiments to ensure the wash protocol was sufficient to clean the tissues of Na^+ (including measurement of the actual Na^+ concentration at the end of each flux period) is required. Further experiments of this type are underway to reexamine the involvement of an NaPi-II-like transport system. It is also possible that the cloned hagfish NaPi-II-like cotransporter may play little to no role in transepithelial movement of inorganic phosphate, but instead is primarily important in phosphate homeostasis at the cellular level. Future research should illuminate these possibilities.

Dogfish

The examination of dogfish salinity tolerance was prompted by broader questions regarding euryhalinity in general and the physiological differences between stenohaline and euryhaline fishes. In particular, the study was designed to detail the time course of homeostatic responses on an ecologically relevant time scale. I found that the North Pacific spiny dogfish is able to tolerate exposure to dilute seawater of 21‰ salinity for at least 48 hours, though I speculated that this is close to the limit of survivable exposure. During the exposure, the animal's rate

of oxygen consumption was elevated between 6-18 hours, and an apparent respiratory acidosis was fully compensated by a metabolic alkalosis. Plasma chloride was stabilized after only nine hours, while sodium decreased considerably more than chloride and did not stabilize until after 12 hours. Plasma urea continuously decreased and showed no signs of stabilization. In fact, measured rates of urea efflux increased over time and remained elevated.

Given the observation that new steady states of homeostasis were achieved for plasma sodium and chloride but not for urea, and based on the results of previous research on this species (Simpson and Ogden 1932), I hypothesized that the minimum level of urea required by the dogfish is the most important factor limiting incursions of this species into brackish water. If directly tested and supported, this would be the first definitive explanation of the limiting factor for an elasmobranch in dilute seawater. Other authors have presumed that the barrier to euryhalinity is a quantitative problem of influx versus efflux of sodium and chloride (Ballantyne and Fraser 2012; Choe et al. 2007; Evans 1984b; Evans 2008), while I present the first hypothesis of urea limitation. Thus, the difference between stenohaline marine elasmobranchs and euryhaline or freshwater elasmobranchs may lie in the ability of the latter to tolerate lower levels of urea in the body. Plasma urea concentrations have been measured as low as 150 mmol l^{-1} in the freshwater-acclimated bull shark (Pillans et al. 2004) and lower than 100 mmol l^{-1} in some species studied by Smith (1931a) and practically zero in freshwater stenohaline rays (Gerst and Thorson 1977). However, Simpson and Ogden (1932) reported that in *S. suckleyi*, the heart would not beat in the presence

of any less than 200 mmol urea l^{-1}. These data are consistent with my urea limitation hypothesis, but definitive measurements of the minimum required level of urea have never been measured except by Simpson and Ogden (1932). Whether similar patterns of urea limitation and the problem of urea balance are reiterated in other stenohaline species may help to form a more general understanding of euryhalinity in elasmobranchs.

NHE2 and NHE3

My observations of adequate sodium and pH homeostasis in dogfish challenged with reduced salinity led to an investigation of the cellular and molecular mechanisms likely responsible for the majority of sodium and acid/base transfers between the environment and the animal. Based on repeated observations of Na^+/H^+ exchange as the major mechanism of both sodium absorption and acid secretion in seawater fishes (*e.g.*, Claiborne et al. (2002), Evans (1982), Heisler (1988)), I pursued a functional analysis of dogfish NHE proteins. I have cloned and sequenced the full NHE2 and NHE3 from the Pacific dogfish gill, stably expressed these in NHE-deficient cell lines, and measured the activity and sensitivity of each NHE isoform to common inhibitory drugs using standard fluorometry techniques. One of my earliest findings was further support for the separation of the North Pacific dogfish and the Atlantic dogfish as separate species based on differences in their NHE2 sequences. Arguably the most important findings of the study were that dfNHE2 is considerably more resistant to amiloride and EIPA than is human NHE2, while dfNHE3 is considerably less

resistant than human NHE3. Furthermore, the dfNHE2 and dfNHE3 isoforms cannot be distinguished from one another by the use of amiloride or EIPA because they have similar sensitivity to these drugs. This is the first examination of inhibitor sensitivity in any non-mammalian NHE2 and NHE3, and the results will be useful in future studies of sodium and pH homeostasis in fishes.

Model for Na^+ uptake and acid excretion via NHE

The proposed functions of apical NHEs in fish gill cells are in transepithelial sodium uptake and acid excretion (Claiborne et al. 1999). This is thought to constitute the major mechanism of sodium influx and the major mechanism of acid excretion for systemic sodium and pH homeostasis (Claiborne et al. 2002).

At the cellular level, the ionocytes or mitochondrion-rich cells of the gill epithelium are the cells most highly enriched in NHEs and other ion transport proteins. Two subtypes of ionocytes have been detected in the elasmobranch gill, one rich in basolateral Na^+/K^+-ATPase (NKA) and one rich in basolateral V-type H^+-ATPase (VHA) (see Figure 5.1). Since the discovery of these two cell types by Piermarini and Evans (2001), the roles of each cell have been the focus of much attention. Based on the localization of various proteins to each cell type and on the mRNA and protein-level responses to homeostatic challenges, it seems that the NKA-rich cells are responsible for acid excretion and sodium uptake, while the VHA-rich cells carry out bicarbonate excretion and chloride uptake (Piermarini and Evans 2002; Tresguerres et al. 2005; Tresguerres et al. 2010).

Both NHE2 and NHE3 have been observed in the apical membrane of NKA-rich cells, while in the VHA-rich cells, NHE3 is absent but cytoplasmic staining for NHE2 has been observed (Choe et al. 2005; Choe et al. 2007; Claiborne et al. 2008). The role of NHE2 in VHA-rich cells is not known. It may translocate to the basolateral membrane and send H^+ back into the blood, or it may have other unknown functions that may not even be related to transepithelial ion transport.

The proposed functions of NHEs in NKA-rich cells are strongly supported by the results of many investigations. While the passive exchange of intracellular H^+ for environmental sodium in dilute freshwater solutions has been questioned on thermodynamic grounds several times (Avella and Bornancin 1989; Kirschner, 2004; Parks et al. 2008), there is no such thermodynamic limitation for NHEs in seawater or brackish water even as dilute as 1% seawater/99% freshwater, owing to the high environmental sodium concentrations and high pH. Only in low sodium freshwater, such as experienced by freshwater stingrays in the Amazon River, are NHEs unlikely to perform.

The functions of the two different NHE isoforms in the same cell are unclear, but data from several species seem to fit a certain pattern. For example, *S. suckleyi* infused with acid showed increased NHE2-like immunoreactivity in western blots of the membrane-enriched fraction of gill homogenates (Tresguerres et al. 2005). The same response was observed in the Pacific hagfish (Parks et al. 2007), and in rainbow trout, NHE2 mRNA increased after hypercapnic acidosis (Ivanis et al. 2008). In the euryhaline stingray, *Dasyatis sabina*, Choe et al. (2005) found gill NHE3 mRNA increased after exposure to reduced salinity but not after

hypercapnic acidosis; NHE2 mRNA was not affected by either treatment. Increased NHE3 expression in response to reduced salinity was also noted by Reilly et al. (2011) in the bull shark *Carcharhinus leucas*. Even in the freshwater zebrafish, evidence thus far implicates NHE3, not NHE2, in sodium uptake (Kumai and Perry 2011; Shih et al. 2012; Yan et al. 2007). In these organisms, NHE2 is apparently recruited in response to acidosis and NHE3 is recruited under reduced sodium conditions. Therefore I propose the following hypothesis: The role of NHE2 in the gill is for transepithelial acid excretion, and the role of NHE3 is for transepithelial sodium uptake. Additional support for this hypothesis was provided by Gens et al. (2007), who observed translocation of rat NHE2 and NHE3 to the cell membrane of PS120 fibroblast cells in response to the respective stressors mentioned above. Potential tests of my hypothesis are discussed below.

Future directions

While the hagfish project was conceived as a stand-alone study, it has opened new avenues of investigation into epithelial transport in the hagfish. Transport of other materials including ammonia and acid/base ions across the intact hagfish skin has been observed (Clifford, Guffey, and Goss, in prep), and the isolated organ methods described in this study can be used as a tool to investigate mechanisms of transport across the gills, skin, and gut. I especially recommend further study using the skin preparation because of its technical simplicity and ease of manipulation. With the addition of a relatively simple set of

electrodes, the transepithelial electrical potential could be measured and active transport of solutes could be monitored under Ussing-type conditions.

The cloning of the hagfish NaPi-II transporter opens up the possibility for a wide variety of functional expression studies. Measurement of the transport kinetics with respect to sodium and phosphate could be readily accomplished using radiotracer and/or electrophysiological techniques in the *Xenopus* oocyte expression system. Furthermore, the effect of sodium-free conditions and sensitivity to phosphonoformic acid could also be determined and this may clarify the question of how much phosphate uptake in the gill and skin actually proceeds through NaPi-II.

The homeostatic response of dogfish to reduced salinity was determined on a whole-animal basis, and several mechanistic questions can easily be pursued. In particular, the hypothesis of urea limitation could be directly tested. By cannulating the caudal artery of dogfish, blood pressure and heart rate can be monitored in vivo before and during exposure to reduced salinity. If heartbeat abnormalities do occur as plasma urea concentration drops, a concentrated solution of urea and/or TMAO can be injected. If one or more of those solutions restores normal heart function, the urea limitation hypothesis will be proven in this species. If gill tissue is harvested after exposure to reduced salinity, expression of NHE2 and NHE3 mRNA and protein can be analyzed to see whether they are upregulated in response to this stressor.

The expression system for the dogfish NHEs that I have established provides a very convenient system for investigating many facets of the function

and regulation of these two isoforms. Future *in vitro* studies, including testing a broader range of putative NHE inhibitors, substrate affinities and molecular determinants of function are all now possible and have not as yet been performed for any fish species. The dfNHE expression system should also be used to test additional inhibitors in hopes of finding some isoform-specific drugs. Promising candidates include cariporide and eniporide for inhibition of NHE3 and S-3226 and BMS284640 for potential inhibition of the NHE2 isoform (Masereel et al. 2003). Also, the sequence data can now be used to create an anti-dogfish NHE3 antibody. In conjunction with the anti-dogfish NHE2 antibody developed by Claiborne et al. (2008), these two antibodies can be tested on my cell lines to determine their specificity. This would be the best possible way to determine whether these antibodies can in fact differentiate these two isoforms and would demonstrate their applicability for later use in immunocytochemistry for cell and tissue localization studies.

Assuming that isoform-specific differential inhibition of NHE2/NHE3 can be achieved, pharmacological means can then be used on whole animals or isolated gills to dissect the functional roles of NHE2 and NHE3 *in vivo*. In acknowledgment of the high price of these drugs, such experiments could be conducted on small dogfish or even gill arches isolated from small dogfish. Free-swimming baby dogfish of 60-100 grams are available in Bamfield from September to November, when they are frequently caught in shrimp trawls. These animals would be ideal for such experiments because ion fluxes can be measured in only one liter of water, and isolated gill arches could potentially be measured in

as little as one milliliter. When I obtained baby dogfish in 2012, I was unable to entice them to feed and few survived more than 10 days, but they proved entirely adequate for solute flux measurements.

Because the sensitivities of dfNHE2 and dfNHE3 to amiloride and EIPA are considerably different from those of mammalian isoforms, it would be prudent to examine the sensitivities of NHEs from other fishes. In particular, the known sequences of trout and zebrafish NHE3s can be expressed and tested with relative ease.

In conclusion, my work has contributed to the understanding of solute homeostasis in hagfish and dogfish, and has also provided some quantitative information on the function of NHE2 and NHE3. Studying solute transport at multiple levels of organization can provide more valuable insight into the functions of proteins, cells, organs, and animals than can be achieved by studying these systems in isolation. In particular, the opportunities afforded by molecular physiology promise to advance the mechanistic study of solute homeostasis in fishes and other organisms, and should be incorporated into more research programs as a complement to, rather than a replacement for, classical physiological studies.

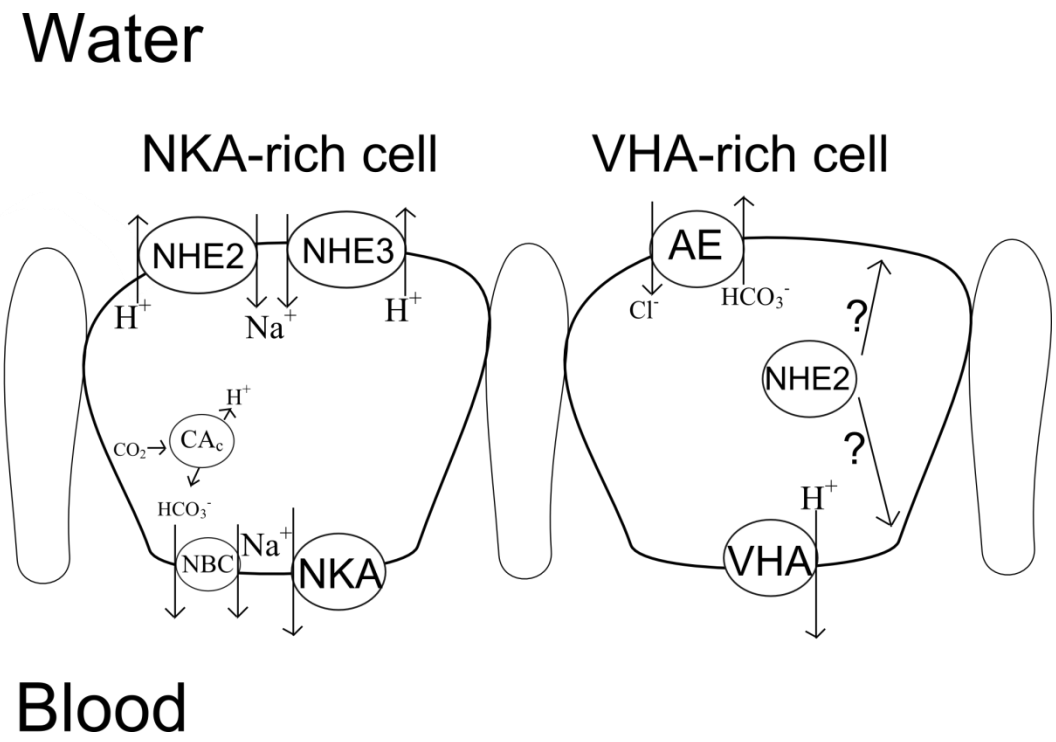

Figure 5.1. **Simplified model of transport mechanisms in NKA-rich and VHA-rich elasmobranch gill cells.** NKA-rich cells express both NHE2 and NHE3 on the apical membrane. I hypothesize that NHE3 is used for transepithelial Na^+ uptake and NHE2 functions primarily for net H^+ excretion. VHA-rich cells are proposed to mediate Cl^- uptake and base excretion; cytoplasmic staining for NHE2 has been observed in *S. acanthias* VHA-rich cells by Claiborne et al. (2008), but the role of NHE2 in this cell is not known. Abbreviations: NKA, Na^+/K^+-ATPase; VHA, V-type H^+-ATPase; AE, pendrin-like anion exchanger; CA_c, cytosolic carbonic anhydrase; NBC, Na^+:HCO_3^- cotransporter.

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