

chapter eleven

*Acid/base and ionic
regulation in hagfish*

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Contents

Introduction	277
Acid/base (A/B) regulation.....	278
Hagfish habitat and behavior: Naturally acidotic environments.....	279
A/B regulatory organs	279
Gills.....	280
Cellular mechanisms for gill A/B regulation.....	281
Responses to metabolic acidosis	284
Responses to metabolic alkalosis	285
Nitrogen balance in the hagfish	286
Nitrogen handling organs.....	287
Gills.....	287
Cellular mechanisms for nitrogen excretion.....	287
Responses to nitrogen challenges	288
Ionoregulation	289
Extrabranchial contributions to acid/base and ionoregulation	290
Concluding remarks	291
Acknowledgments	291
References	292

Introduction

Hagfish are the living representative of the most basal craniate and share an ionic profile similar to that seen in marine invertebrates, representing the evolutionary transition between invertebrate and vertebrate organisms. It is these unique physiological and evolutionary positions that make hagfish an excellent model to study the evolution of acid/base (A/B) and ionic regulation. Hagfishes diverged from the main vertebrate lineage more than 500 million years ago with fossil evidence supporting that their

morphology has remained unchanged for ~450 million years (Holland and Chen, 2001). Hagfishes are exclusively osmoconforming marine animals with most living at considerable depths and are the only living vertebrates to maintain their plasma $[\text{Na}^+]$ to $[\text{Cl}^-]$ almost iso-osmotic to that of seawater (Hardisty, 1979). Coupling the unique osmoregulatory characteristics of hagfish along with the fact that fossil evidence comes solely from marine-based sediments, it may be inferred that hagfish never entered a freshwater environment. Considering that their morphology has remained relatively unchanged, it can be hypothesized that their current physiology may reflect the physiology of vertebrate ancestors prior to their invasion of estuaries, freshwater, and land (Hardisty, 1979; Holland and Chen, 2001).

Hagfish are stenohaline osmoconformers who maintain their blood osmolarity slightly hypertonic in comparison to seawater. They have no ability or need to regulate plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$, but they have been shown to regulate $[\text{Ca}^{2+}]$, $[\text{Mg}^{2+}]$, and $[\text{SO}_4^{2-}]$, which are hypo-regulated (Bellamy and Jones, 1961). Despite the inability to regulate $[\text{Na}^+]$ and $[\text{Cl}^-]$, a number of studies have identified genes associated with ion transport and acid/base regulation (Karnaky, 1998; Choe et al., 1999, 2002; Edwards et al., 2001; Tresguerres et al., 2006a).

Acid/base (A/B) regulation

All fishes including hagfish must maintain internal pH homeostasis, as the concentration of H^+ (~pH) affects the kinetics of chemical reactions and the structure and function of proteins in all organisms (Whitten et al., 2005; Srivastava et al., 2007). Fish, similar to terrestrial vertebrates, use a strategy of buffering and excretion to defend against both intracellular and extracellular pH alterations. The processes of intracellular metabolism generate free H^+ and HCO_3^- from CO_2 , following the Henderson–Hasselbalch equation (reviewed by Claiborne et al., 2002). Although the intracellular buffering capacity in hagfishes is not well characterized, in higher fishes both inorganic and organic phosphates, proteins, and amino acid residues have been shown to limit free H^+ . However, to maintain intracellular pH, critical for proper cellular function, cells must additionally actively import and export H^+ and HCO_3^- via transport proteins located in the cell membrane (Roos and Boron, 1981). The ability of fishes to compensate for metabolic and environmental disturbances is limited by the aquatic environment in which they reside, and therefore most fishes regulate blood pH by secreting and absorbing excess H^+ and HCO_3^- across specialized epithelia such as gills and skin (reviewed by Perry, 1997). Other factors that help maintain or affect extracellular fluid (ECF) and blood A/B homeostasis are buffer systems, such as $\text{CO}_2/\text{HCO}_3^-$, $\text{NH}_3/\text{NH}_4^+$, phosphates, and proteins (Heisler, 1986; Boron, 2004).

Hagfish habitat and behavior: Naturally acidotic environments

Hagfish are demersal and either burrow into the sediment (genus *Myxini*) or reside on top of the ocean bed (genus *Eptatretus*) (Martini, 1998). These environments are usually quite hypoxic or even anoxic (McInerney and Evans, 1970; Smith and Hessler, 1974) and likely determine the low metabolic rates observed in hagfish (Munz and Morris, 1965; Forster, 1990). Hagfish also have a high capacity for anaerobic respiration (Sidell et al., 1984), which can lead to metabolic acidosis.

Hagfish have traditionally been considered to be scavengers; however, recently they have been observed to also actively hunt live prey, dragging the victim back into their burrows (Zintzen et al., 2011). Hagfish consume a wide variety of prey types including polychetes, squid, shrimp, crab, and teleosts (Shelton, 1978), sometimes robbing food captured by other fauna (Auster and Barber, 2006). Active hunting behavior is likely associated with burst swim activity potentially causing acute blood acidosis (Ruben and Bennett, 1980). However, hagfish are able to readily recover from such energetic bouts, with little disturbance to their blood A/B status (Davison et al., 1990).

Hagfish are best known for their opportunistic scavenging behavior, as they have been regularly reported to feed on dead and decaying carrion that falls to the ocean floor (Smith, 1985; Martini, 1998; Collins et al., 1999; Auster and Barber, 2006). Sources of carrion may include by-catch from commercial fishing (Collins et al., 1999) or expired large mammals (Smith and Baco, 2003), but the most likely source is leftovers from other predator-prey interactions. Hagfish burrow into the decomposing carcass (Jensen, 1966; Martini, 1998), which presents both opportunities and challenges. As the carcass is being consumed, decomposition of the tissues by bacteria is believed to generate an environment that is rich in free amino acids, which hagfish may absorb across gills and skin (Glover et al., 2011; Chapter 13). However, the metabolism of bacteria, the hagfish itself, and other animals feeding inside carcasses likely also results in elevations in PCO_2 , PNH_3 , $[H^+]$, and weak acids such as butyric acid, putrescine, and formic acid, which are known to cause acute acidification of tissues.

A/B regulatory organs

The gill is the main blood A/B regulatory organ in adult fish (Evans et al., 2005), and certain areas of the skin fulfill this role in larval fish before gills develop (Wells and Pinder, 1996). Similarly, gills were considered as the main A/B organ in hagfish (Evans, 1984; Edwards et al., 2001); however, recent research has demonstrated that the skin, especially in the posterior body, also plays an important role in blood A/B homeostasis (Clifford et al., 2014). Although the renal system in more derived aquatic species

(e.g., Chondrichthyes, Osteichthyes) plays a distinctive role in A/B regulation (Claiborne et al., 2002), the hagfish renal output is too low (0.1–1.0 mL 100/g/day; Hardisty, 1979) to significantly contribute to acute A/B regulation. Nonetheless, hagfish kidneys have been shown to resorb Na^+ , which may play a significant role in nutrient uptake from the filtrate (McInerney, 1974; discussed below).

Gills

The gill pouches of the hagfish are morphologically different compared with gills from lampreys, Chondrichthyan fishes, and Osteichthyan fishes (Figure 11.1). Hagfish pouches are found along the length of the anterior half of the body and range from 6 to 13 pairs in number displaying both intra- and interspecific variability (Rauther, 1937; Chapter 1). All gill pouches are morphologically similar and consist of a muscular wall lined up with an epithelium known as the “primary filament,” from which multiple “lamella”

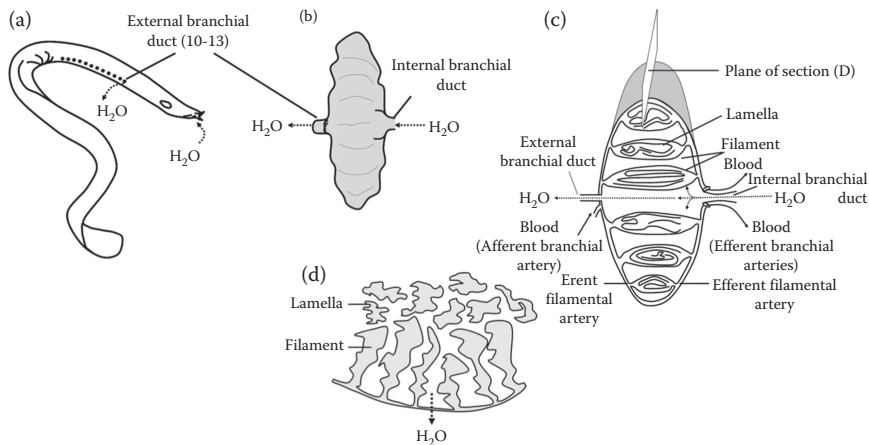


Figure 11.1 Schematic of the hagfish gill pouch gross anatomy and fine structure. (a) Water enters the hagfish through the nasopharyngeal duct and leaves across the 6–13 pairs of gill pouches located at each side of the body. (b) External morphology of a gill pouch. (Modified from Mallatt, J. and C. Paulsen. 1986. *Am J Anat* 177:243–269.) Water and blood flow in counter-current fashion. The surface area of filaments and lamella is enlarged by radial infoldings, as opposed to the typical structures found in lampreys, elasmobranchs, and teleosts. The plane of the section used in the immunohistochemistry study is illustrated on top of the pouch, which is also shown in more detail in (d). (d) Section across the gill pouch in the orientation indicated in (c). The gill filaments and lamella are shown in the same view as the immunohistochemistry sections of Figure 11.3. Notice how the radial infolding of the lamella results in an apparent separation from the filaments in this plane of section. (Adapted from Tresguerres et al. 2006a. *Comp Biochem Physiol A* 145:312–321.)

radiate toward the pouch lumen (Mallatt and Paulsen, 1986). Whether or not all gill pouches are also functionally identical has not yet been discerned.

Ultrastructural studies on hagfish gills have revealed the presence of pavement, basal and intermediate cells, and ionocytes (Elger and Hentschel, 1983; Bartels, 1984, 1985; Mallatt and Paulsen, 1986; reviewed in Chapter 1). Ionocytes, cells characterized as possessing large number of mitochondria, typically support transepithelial ATPase-driven ion-transporting processes. The ionocytes in hagfish gills have been shown to express ion transporter mRNAs and proteins associated with the excretion and absorption of H^+ and HCO_3^- for A/B regulation (Evans, 1984; Mallatt et al., 1987; Choe, 1999; Edwards et al., 2001; Tresguerres et al., 2007a; Parks et al., 2007b) and have also been demonstrated to uptake inorganic phosphate (Schultz et al., 2014) and amino acids (Glover et al., 2011).

Cellular mechanisms for gill A/B regulation

In all organisms, transepithelial A/B regulation is achieved by the combined action of various ion-transporting proteins differentially expressed in the basolateral or apical membranes, or in the cytoplasm; the overall result is excretion of excess H^+ and absorption of HCO_3^- to correct blood acidosis, and excretion of excess HCO_3^- and absorption of H^+ to correct blood alkalosis. In marine organisms, the most typical ion-transporting proteins involved in A/B regulation are Na^+ - K^+ -ATPase (NKA, located in the basolateral membrane), carbonic anhydrase (CA, cytoplasmic and membrane-bound), Na^+ / H^+ exchangers (NHEs; HUGO gene family SLC9), Na^+ / HCO_3^- cotransporters (NBCs; HUGO gene family SLC4), and Cl^- / HCO_3^- exchangers (CBEs; HUGO gene family SLC26) (with the latter three located in either apical or basolateral membrane, depending on the specific isoform and cell type). Another relevant protein is the V-type H^+ -ATPase (VHA), in which intracellular localization and specific role varies depending on the species, cell type, and A/B condition (for review, see Evans et al., 2005).

In a generalized model, CO_2 enters the ionocytes from blood plasma (through a mechanism that may depend on plasma membrane-bound CA) and is also produced in mitochondria. Once in the ionocyte, cytoplasmic CA catalyzes its hydration into H^+ and HCO_3^- , which are subsequently differentially excreted to seawater or absorbed into the blood, depending on the A/B regulatory needs. To compensate for blood acidosis, H^+ are excreted to the surrounding seawater in exchange for Na^+ through apical NHEs; this transport is secondarily energized by basolateral NKA (Evans et al., 2005). The proteins responsible for HCO_3^- absorption are not as well characterized, although suitable candidates are basolateral CBEs and NBCs (Parks et al., 2007a). To compensate for blood alkalosis, HCO_3^- is excreted in exchange for Cl^- through apical CBEs. However, the rest of the mechanism is much less understood and may vary considerably from

species to species. The driving force may be provided by NKA, for example, by creating a cell membrane potential that favors a low intracellular $[\text{Cl}^-]$, or by linkage of apical $\text{Cl}^-/\text{HCO}_3^-$ exchange with Na^+ uptake (e.g., Na^+ -dependent CBEs) (Parks et al., 2009). In this case, H^+ transport from the ionocyte into the blood most likely takes place by basolateral NHEs, as described in the intestine of marine fish (Grosell and Genz, 2006) and crab gills (Tresguerres et al., 2008).

Another option involves basolateral VHA as described in shark gill ionocytes; in this mechanism, VHA “pumps” H^+ into the blood, which drives apical $\text{Cl}^-/\text{HCO}_3^-$ exchange by removing H^+ from the cytosol (thus preventing CA inhibition and reversal by law of mass action) and by promoting Cl^- absorption into the blood across channels in the basolateral membrane (due to the membrane potential generated by H^+ transport) (Tresguerres et al., 2006b). Regardless of the specific mechanisms, lampreys, elasmobranchs, and bony fish have developed gill ionocyte subtypes specialized for acid or base secretion (reviewed in Evans et al., 2005). Hagfish follow the general model of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange across the gill epithelium (Evans, 1984: Figure 11.2a). However, histochemical and immunohistochemical evidence suggests that hagfish gills have a single type of ionocyte that alternatively performs H^+ or HCO_3^- secretion depending on the blood A/B condition (Tresguerres et al., 2006a). The vast majority of experiments on hagfish A/B regulation have been conducted on either the Atlantic hagfish (*Myxini glutinosa*) or the Pacific hagfish (*Eptatretus stoutii*) with little information on the other species (e.g., *E. burgerii*). Based on the published results, this chapter assumes that the cellular mechanisms for A/B regulation are similar for all hagfish species; for details about the experimental species used in each study, the reader is directed to the cited literature.

Hagfish decrease net H^+ or HCO_3^- secretion when placed in Na^+ - or Cl^- -free seawater, respectively (Evans, 1984). Given that hagfish represent the only chordate group assumed to have never entered freshwaters (Hardisty, 1979), the presence of these transport mechanisms has led to the hypothesis that these mechanisms evolved for pH balance prior to their use in ionoregulation and that these mechanisms were co-opted for Na^+ and Cl^- absorption for ionoregulation by the chordates that originally invaded estuarine and freshwater environments (Evans, 1984).

The first suggestion that hagfish gill ionocytes are involved in blood A/B regulation was biochemical evidence of NKA and CA activities in histological sections of hagfish gill pouches (Mallatt et al., 1987). Two subsequent studies used immunohistochemistry to localize NKA and NHEs in hagfish gill epithelium (Choe et al., 1999, 2002), and a third study suggested co-localization of NKA, $\text{V-H}^+\text{ATPase}$, and an NHE2-like in a single gill cell type (Tresguerres et al., 2006a). The anti-NKA and anti-VHA antibodies used in those studies were raised against amino

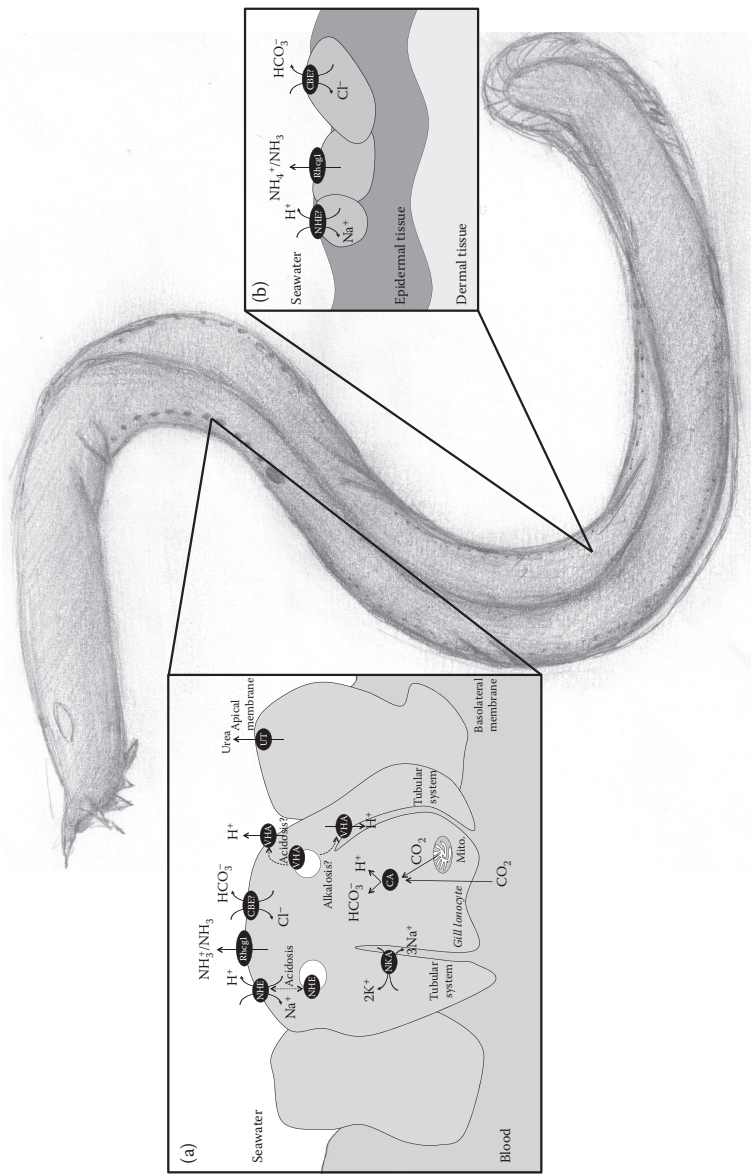


Figure 11.2 Molecular components of hagfish acid-base and ionoregulation in (a) gill and (b) skin. NKA, Na^+/K^+ -ATPase; VHA, V-H^+ -ATPase; NHE, Na^+/H^+ exchanger; RHCg1, Rh glycoprotein c1; UT, urea transporter; CBE, chloride bicarbonate exchanger; CA, carbonic anhydrase.

acid regions that are highly conserved in all animals and therefore there is little doubt they specifically recognize hagfish NKA and VHA proteins. However, the anti-NHE antibodies used were against mammalian NHE isoforms that are not as well conserved in other animals, so these results should be interpreted with caution. Another potential limitation of Tresguerres et al. (2006a) was that colocalization of NKA, VHA, and NHE2 was inferred from consecutive histological gill sections, which do not always contain the same cells (in fact, colocalization was not evident in all sections examined). However, more recent dual immunolocalization of NKA and VHA on the same gill section confirms that these two proteins are indeed present in the same cells (Figure 11.3).

Responses to metabolic acidosis

Hagfish have an outstanding capacity for recovering from metabolic acidosis; this has been determined from experiments that induced blood

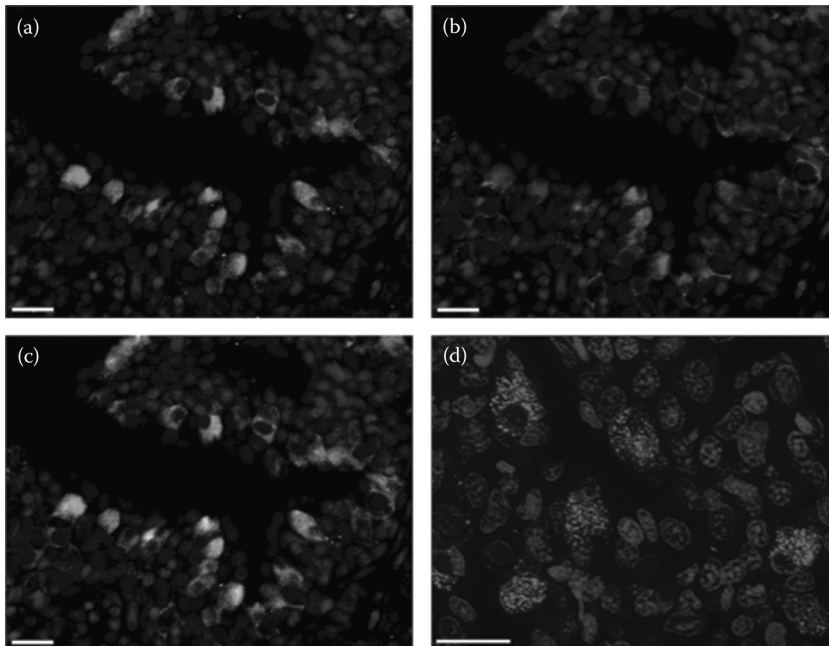


Figure 11.3 (See color insert.) VHA- and NKA-rich cells in hagfish gills. Dual immunolocalization of VHA (a, green) and NKA (b, red), with VHA and NKA immunoreactivity found in the same gill cells (c). At higher magnification with optical sectioning, NKA immunoreactivity was visible as punctate staining throughout the edge of cell, indicative of NKA localization along the basolateral membrane (d). Nuclei in blue. Scale bar: 20 μ m.

acidosis by injections of H_2SO_4 (3 mequiv. H^+ /kg; McDonald et al., 1991; Edwards et al., 2001) or HCl (6 mequiv. H^+ /kg; Parks et al., 2007b) into the posterior caudal blood sinus. Blood pH suffered initial sharp reductions between ~ 0.6 (McDonald et al., 1991) and up to ~ 1.6 pH units (Parks et al., 2007b). However, blood pH was fully (McDonald et al., 1991) or near fully (Parks et al., 2007b) rectified within 6 h, demonstrating a significant capacity for rapid A/B regulation. In the latter study, subsequent injections caused significantly smaller reductions in blood pH compared with the initial injection, indicating increased capacity for acid secretion upon sustained acidosis. Metabolic acidosis resulted in upregulation in gill NHE2-like mRNA (Edwards et al., 2001) and protein abundance (Parks et al., 2007b), as well as protein insertion in the apical membrane of gill ionocytes (Parks et al., 2007b) (Figure 11.2a). On the other hand, the protein abundance of NKA and VHA remained unchanged (Parks et al., 2007b). These results indicate that apical NHEs are involved in acid secretion and suggest that the basal levels of NKA are sufficient to sustain upregulated acid secretion. However, other NKA-activating mechanisms (NKA isoforms, phosphorylation) cannot be ruled out.

Responses to metabolic alkalosis

Little research has been conducted evaluating hagfish responses to metabolic alkalosis. It is also less clear which conditions would result in blood alkalosis in the wild. One possibility could arise following feeding in a hypercapnic environment (e.g. decaying carcass) where the resultant blood acidosis is compensated by increases in plasma $[\text{HCO}_3^-]$ as has been described in other fishes (Brauner and Baker, 2009). Another potential source of blood alkalosis is a postprandial alkaline tide as observed in elasmobranchs (Wood et al., 2005; Tresguerres et al., 2007b), and both freshwater (Bucking and Wood, 2008) and seawater-acclimated trout (*Oncorhynchus mykiss*) (Bucking et al., 2009). However, postprandial digestive strategies of hagfishes remain to be identified.

Nonetheless, it is clear that hagfish possess efficient mechanisms to rectify blood metabolic alkalosis (Tresguerres et al., 2007a). Injection of base (NaHCO_3 ; 6 mequiv. HCO_3^- /kg) caused sharp increases in blood pH and plasma $[\text{HCO}_3^-]$ and both were rectified within 6 h. Similar to the acid injections described above, the impact of subsequent base injections on blood pH was diminished, indicating capacity for upregulation of base-secretory mechanisms. After four NaHCO_3^- injections at 6 h intervals (24 h), there was an increase in VHA protein abundance both in gill homogenates and in enriched gill cell membrane fractions, estimated from Western blots. As accumulation of VHA in the apical membrane was not evident in immunostained gill sections, these results suggest a translocation of VHA to the basolateral membrane to counteract blood alkalosis as

described in dogfish sharks (Tresguerres et al., 2005). However, the tubulovesicular system in hagfish gill ionocytes complicates differentiating between cytoplasm and basolateral membrane localization using immunohistochemistry techniques, unlike shark ionocytes that lack a tubulovesicular system (e.g., see Tresguerres et al., 2005). Although there were no significant changes in NKA abundance in gill homogenates, a reduction in NKA abundance was detected in enriched gill cell membrane fractions, also by Western blot. As hagfish gills possess only one ionocyte subtype that is rich in both NKA and VHA, it was postulated that the differential activation of acid and base secretion occurs via differential insertion of NKA and VHA into the basolateral membrane and of NHE and an as of yet unidentified CBE into the apical membrane (Tresguerres et al., 2006a, 2007a; Parks et al., 2007b). In addition, a recent study has suggested that, at least at shorter time points (4 h), extrabranchial mechanisms are more important than gills for recovering from blood alkalosis (Clifford et al., 2014; see "Extrabranchial contributions to acid/base and ionoregulation").

In all other chordates studied, there exist two or more ionocyte subtypes (Doyle and Gorecki, 1961; Lin et al., 2006), with distinct utility for regulating acid/base status (Reid et al., 2003; Goss et al., 2011). In the case of lampreys and elasmobranchs, acid and base secretion is likely split between two subtypes: NKA-containing and V-H⁺ATPase-containing (Piermarini and Evans, 2001; Piermarini et al., 2002; Choe et al., 2004; Tresguerres et al., 2005, 2006b). The same is true for teleost fish, where at least two or more different subtypes have been identified with at least one subtype capable of acid secretion and another for base secretion (for review, see Hwang and Lee, 2007; Hwang et al., 2011). The presence of a single ionocyte type in hagfish suggests that it is an ancestral character and that more derived groups evolved multiple ionocyte subtypes in relation to novel environmental and metabolic challenges (Tresguerres et al., 2007a).

Future studies on base-secreting mechanism in hagfish should explore the relative contributions of branchial and extrabranchial mechanisms upon acute and chronic blood alkalosis, identify the putative apical CBE(s) involved, as well as the regulatory mechanisms that allow switching from acid to base secretion. To the latter aim, optimization of techniques such as isolated gill pouches and primary cultures of isolated gill cells would be beneficial, as they allow measuring H⁺ and HCO₃⁻ fluxes and intracellular pH dynamics, respectively.

Nitrogen balance in the hagfish

Build-up of ammonia (NH₃) and ammonia-related compounds within organisms is toxic, causing a variety of impairments including disruption of biochemical pathways, interference of ionic movement across

transmembrane surfaces via ion substitution of other monovalent ions such as Na^+ and K^+ , and disruption of extracellular and intracellular pH via the strongly basic NH_3 (Walsh, 1998). The high protein diet of hagfishes (Smith, 1985; Martini, 1998; Collins et al., 1999; Auster and Barber, 2006) results in uptake of a large amount of amino acids. Catabolism of these ingested amino acids not only supplies the carbon backbone necessary for conversion into fatty acids, and carbohydrates (Nelson et al., 2008), but also contributes to the net nitrogen load of the fish, which must be excreted (for excellent reviews, see Wood, 2001; Evans et al., 2005). Other sources of nitrogen include the deamination of protein during fasting periods or, perhaps more relevant to the scavenging behavior of the hagfishes, from a decaying food source. No data exists on the physicochemical characteristics inside or surrounding decaying carrion in aquatic environments, however Clifford et al. (2015) argue that such an environment would be similar to decomposition in terrestrial environments where putrefactive gases (hydrogen sulfide and ammonia) generated by anaerobic microflora lead to high deposits (~ 30 mmol/L) of nitrogenous compounds in the surrounding soil (Carter et al., 2007). Such deposits in an aquatic environment would present high inwardly-directed ammonia gradients to an encapsulated animal during a prandial event.

Nitrogen handling organs

Gills

The gills are a primary site of NH_3 exchange for many aquatic vertebrates (Wood, 1993; Wright et al., 1995) and most aquatic invertebrates (Henry et al., 2012). Additionally, various strategies exist to combat high ammonia such as ureotelosis as in the lake magadi tilapia (*Oreochromis alcalicus grahami*; Randall and Wright, 1989) and Gulf toadfish (*Opsanus beta*; Walsh et al., 1990), glutamine production in the swamp eel (*Monopterus albus*; Ip et al., 2010), and active NH_4^+ excretion used by the mudskipper (*Periophthalmodon schlosseri*) while burrowed (Randall et al., 1999; Wilson et al., 2000). Recently, hagfish have been shown to have low basal rates of $\text{NH}_3/\text{NH}_4^+$ and urea excretion, with both the gills and the skin being hypothesized as sites of passive excretion (Braun and Perry, 2010; Clifford et al. 2015).

Cellular mechanisms for nitrogen excretion

Despite reported low basal rates of nitrogenous waste excretion, hagfish have remarkable ability to excrete excess nitrogen taken up by either exposure to high environmental ammonia (HEA; Clifford et al., 2015) or via direct infusion of NH_4Cl (McDonald et al., 1991; Edwards et al., 2015) or urea (Braun and Perry, 2010). Transcellular transport of ammonia and urea may be, respectively, facilitated by Rh glycoproteins (Marini

and Urrestarazu, 1997) and urea transporter (UT) proteins (Levine et al., 1973a, b; You et al., 1993). The Rh glycoproteins are homologs of the Amt superfamily and have multiple isoforms (Huang and Peng, 2005), however the isoforms of interest to fish physiology and ammonia excretion are Rhag, Rhbg, and Rhcg (Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007; Tsui et al., 2009). In gills from the marine teleost *Takifugu rubripes*, Rhag localizes to the apical and basolateral membranes of the pillar cells and mediates ammonia transport from red blood cells through pillar cells (Nakada et al., 2007). Rhbg localizes to the basolateral membrane of pavement cells, whereas Rhcg2 localizes to the apical membrane. Finally, Rhcg1 localizes on ionocytes on apical, gill/water interface. However, teleost fishes are highly derived compared with the hagfishes, so the roles and localizations of any hagfish-specific Rh glycoproteins may differ.

To date, two Rh glycoprotein isoforms from hagfish have been cloned, Rhbg and Rhcg, and are expressed in hagfish gill and skin (Edwards et al., 2015) with the presence of Rhag so far remaining elusive. Hagfish-specific Rhcg protein and mRNA expression have been demonstrated in both gill and skin tissues. In gill, Rhcg is localized to the basal aspect of the filament epithelium along the region of the filament closest to the blood margin and in the skin hRhcg immunoreactive cells were localized to regions surrounding mucous glands (Edwards et al. 2015). In addition, using heterologous antibodies and immunohistochemistry, Braun and Perry (2010) found Rhbg localized to the lining of larger blood vessels (i.e., noncapillary vessels), whereas Rhcg1 colocalized apically to NKA-enriched gill cells. Localization of Rhbg to the blood vessels points to a different role for this isoform in hagfish, likely mediating transcellular transport of ammonia from tissues to the blood (Braun and Perry, 2010).

Responses to nitrogen challenges

Basal ammonia excretion rates for hagfish are about 40–60 $\mu\text{mol kg}^{-1} \text{h}^{-1}$ (Braun and Perry, 2010; Clifford et al., 2014, 2015), half that of most ammonotelic fishes (100–350 $\mu\text{mol kg}^{-1} \text{h}^{-1}$; Wood, 2001; Evans et al., 2005) and on par with larval and parasitic sea lamprey (*Peteromyzon marinus*; 20–50 $\mu\text{mol/kg/h}$; Wilkie et al., 1999, 2004) and the ureotelic elasmobranchs (~50 $\mu\text{mol/kg/h}$; reviewed by Wood, 2001). Multiple studies have described the ability of the hagfish to survive high inwardly-directed ammonia gradients with survival reported following 9 h exposure to 100 mM NH_4Cl in seawater (Braun and Perry, 2010) and 48–72 h exposure to 20 mM NH_4Cl (Clifford et al., 2015). These studies have also highlighted the capacity of hagfish to tolerate extremely high plasma ammonia loads with the highest reported plasma ammonia load peaking at 5450 μM following 24 h exposure to 20 mM HEA (Clifford et al., 2015). Remarkably, during continued

exposure to HEA, hagfish are able to excrete ammonia against massively inwardly-directed gradients, thus stabilizing rates of uptake (Clifford et al., 2015). During recovery from HEA exposure, hagfish are capable of excreting ammonia/ammonium at high rates ($\sim 3000 \mu\text{mol/kg/h}$; Clifford et al., 2015). The high rate of ammonia offloading is comprised of both branchial and extrabranchial transport with the branchial contribution comprising approximately 70–80% and excretion through the skin comprising the remainder (Clifford et al., 2014, 2015; see “Extrabranchial contributions to acid/base and ionoregulation”).

A recent study where hagfish were injected with $3 \text{ mmol/kg NH}_4\text{Cl}$ demonstrated that elevated plasma ammonia concentrations paralleled elevated ammonia excretion rates and coincided with initial significant upregulation of Rhcg mRNA expression in the gill and the skin, suggesting that the transcriptional regulation of Rh glycoproteins may respond to elevated plasma ammonia. The study also used homologous antibodies to demonstrate the subsequent significant upregulation of hagfish Rhcg protein, suggesting that Rh glycoproteins are involved in the regulation of ammonia excretion in hagfish (Edwards et al., 2015).

Basal excretion of urea in hagfish has been reported to be $\sim 30 \mu\text{mol/kg/h}$ (Braun and Perry, 2010), again on par with excretion rates observed in parasitic lamprey (Wilkie et al., 2004). In *E. stoutii*, urea excretion did not change during exposure to HEA (10–100 mM) and during recovery in nominally ammonia-free seawater (Braun and Perry, 2010; Clifford et al., 2015). However, following infusion of NH_4Cl that raised plasma ammonia to 10 mM, threefold increases in urea excretion were observed following a 6-h period. Direct infusion of urea (to raise plasma urea to $\sim 100 \text{ mM}$) caused an immediate (0–3 h) 1000-fold surge in urea excretion which tapered to ~ 250 -fold compared with control animals (Braun and Perry, 2010).

Ionoregulation

Similar to A/B balance, the maintenance of stable plasma ionic composition is crucial for normal physiological function. Ionic regulation is traditionally associated with Na^+ and Cl^- transport because these are the two major constituents of internal fluids and therefore are the major contributors to total osmolarity. However, plasma ions such as K^+ , Ca^{2+} , Mg^{2+} , and SO_4^{2-} are also maintained at significantly lower concentrations compared with the environment; these are especially relevant for hagfish plasma as they are the only ions whose concentrations are significantly lower than seawater (Currie and Edwards, 2010). Ions that contribute to A/B balance (e.g., H^+ , HCO_3^- , NH_4^+) are discussed in previous sections.

Ionic gradients between internal fluids and the marine environment originated in the first unicellular organisms; these gradients were

essential for processes such as energy generation, uptake of molecules from the environment, and counteraction of negatively charged proteins (Yancey, 2005). As an ionic gradient is associated with water fluxes that can disrupt cells by swelling and shrinking, cells also accumulate other solutes such as proteins, amino acids, and other osmolytes to bridge the osmotic gap with the environment.

The osmolarity of hagfish plasma is essentially the same as in seawater (Smith, 1932) and therefore hagfish do not experience major water fluxes with the environment; this strategy is called “osmoconforming.” Later works demonstrated that hagfish plasma is also similar to seawater in ionic composition (Robertson, 1954; Bellamy and Jones, 1961), with the exception of Ca^{2+} , Mg^{2+} , and SO_4^{2-} (Bellamy and Chester Jones, 1961). Due to the similarity of osmotic strategies, hagfish have been considered to have more in common with invertebrates than the vertebrates (Currie and Edwards, 2010). Indeed, Na^+ and Cl^- are the major osmolytes in hagfish plasma which lack the capacity to regulate plasma osmolality, $[\text{Na}^+]$, and $[\text{Cl}^-]$ (Sardella et al., 2009), similar to most marine invertebrates. The strict osmoconforming strategy likely reflects an exclusive evolutionary history of hagfish in seawater and it can also explain why there are no fossil records of hagfish in sediments other than of marine origin and why there are not extant estuarine or freshwater hagfishes.

Although plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ are similar to seawater, plasma $[\text{Ca}^{2+}]$, $[\text{Mg}^{2+}]$, and $[\text{SO}_4^{2-}]$ are regulated to levels one-half to one-third that of seawater (Robertson, 1954, 1976; Bellamy and Jones, 1961). For example, unlike $[\text{Na}^+]$ and $[\text{Cl}^-]$ (Sardella et al., 2009), hagfish can actively regulate $[\text{SO}_4^{2-}]$ in the absence of any changes in glomerular filtration rate (Clifford et al., unpublished). Similarly, information about mechanisms for active regulation of plasma $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ is still lacking. Given the phylogenetic position of the hagfish and its ability to regulate these divalent ions but not plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$, it seems that ionoregulation of divalent ions evolved prior to regulation of monovalent ions. However, validation of this hypothesis requires further study examining the ionoregulatory capacities of organisms from major clades ancestral and derived in relation to the agnathans.

Extrabranchial contributions to acid/base and ionoregulation

Recent developments in hagfish physiology have begun to highlight the extrabranchial aspects of acid/base and ionoregulatory status, with specific regard to the skin performing nutrient acquisition. Glover et al. (2011) proposed that the feeding environment (i.e., putrefied carrion) of the hagfish would be high in amino acid content and that the hagfish may have

adapted to take up nutrients across multiple epithelia. The group hypothesized that while burrowing into a carcass to feed, hagfish optimize nutritional uptake via amino acid transport through both the skin and gill. The findings from this study did indeed show *in vitro* uptake of L-alanine and L-glycine through the intestine and more importantly in the gills and the skin (Glover et al., 2011; see Chapter 12), thus highlighting the adaptations that hagfish have acquired to maximize nutrient uptake while burrowing into food sources. The intestine, skin, and gills of hagfish have also recently been shown to facilitate uptake of inorganic phosphate, a limiting nutrient in the demersal habitat of the hagfish (Schultz et al., 2014; reviewed in Chapter 12).

In addition to being nutrient-rich, the environment in and around the putrefying carrion is also likely high in PCO_2 , PNH_3 , and acidic pH (see “Hagfish habitat and behavior: Naturally acidotic environments”). Presumably, hagfish have developed similar extrabranchial adaptations in ionoregulatory capacity to cope with these environmental stressors when hagfish burrow into carrion (Jensen, 1966; Martini, 1998). Anecdotally, we have noticed that while feeding, the posterior portion of the animal often remains outside the carrion. Thus, the caudal region is immersed in seawater that is nominally free from putrefactive gases and would therefore present favorable gradients to offload toxicants (excess NH_4^+ , NH_3 , H^+ , HCO_3^- , CO_2). We are currently investigating the potential role of extrabranchial tissues (specifically the skin, intestine, and kidney) in ionoregulation, A/B regulation, and nitrogen handling as unique adaptations to the hagfish feeding behavior/strategy.

Concluding remarks

Although the description of the unique ionoregulatory strategy of hagfishes has been known for more than 85 years (Smith, 1932), the molecular and cellular mechanisms have only recently begun to be described. The recent development of advanced sequencing approaches combined with strategies/techniques for understanding cellular mechanisms of ion and acid–base regulation have resulted in a resurgence of interest in hagfish as a key evolutionary species. Future developments in our understanding should provide valuable new information on the physiology of this unique organism and will aid in our understanding of the evolution of ionoregulatory and acid–base regulatory systems.

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