J Comp Physiol B (2009) 179:721–728
DOI 10.1007/s00360-009-0355-3

ORIGINAL PAPER

The effects of variable water salinity and ionic composition on the plasma status of the Pacific Hagfish (*Eptatretus stoutii*)

B. A. Sardella · D. W. Baker · C. J. Brauner

Received: 4 November 2008 / Revised: 19 February 2009 / Accepted: 19 March 2009 / Published online: 7 April 2009

Abstract Hagfish are the most pleisiomorphic extant craniates, and based on the similarity of ionic concentrations between their internal milieu and seawater (SW), they have long been touted as a model for osmo- and ionoconformation. As a result, the lack of direct symmetry between hagfish plasma and the environment with respect to [Na^+], [Cl^-], [Mg^{2+}], and [Ca^{2+}] have been left largely unexplored. In order to determine the capacity of hagfish to regulate their blood compartment, we exposed Pacific hagfish (*Eptatretus stoutii*) to 24, 32, 40, and 48 g/l salinity for 48 h, as well as to two treatments where a portion of the water [Na^+] was replaced with either Mg^{2+} or Ca^{2+} at constant salinity for up to 6 days. Following exposure, we measured plasma ion status, pH, and total carbon dioxide (TCO_2). As expected, our results indicated that hagfish had no capacity to regulate plasma osmolality, [Na^+], or [Cl^-], but they did maintain plasma [Mg^{2+}] and [Ca^{2+}] nearly constant despite fluctuation of environmental salinity or elevated water [Mg^{2+}] and [Ca^{2+}] (two- and sevenfold, respectively). Furthermore, exposure to elevated water [Mg^{2+}] and [Ca^{2+}] resulted in a large increase of plasma TCO_2 with little to no increase of plasma pH. We concluded that hagfish may control plasma [Mg^{2+}] and [Ca^{2+}] at levels below that of their environment via secretion of HCO_3^-, similar to the mechanisms described in the intestine of teleosts. We speculate that secretion of HCO_3^- likely evolved to maintain plasma [Mg^{2+}] and [Ca^{2+}] below environmental levels (both of which negatively affect nervous function and muscle contraction if elevated), and was an exaptation for the development of water-absorption mechanisms in the intestine of marine osmoregulators. The ancestors of modern hagfish are thought to have never entered freshwater, thus investigations into their ionoregulatory ability potentially have profound implications regarding the evolution of fishes.

Keywords Hagfish · Salinity · Ion regulation

Introduction

Hagfishes (Myxinoides) are the most pleisiomorphic extant craniates and are thought to share a common ancestry with all other fishes (Janvier 2001; Wright 2007). Smith (1932) was the first to note that the osmolality and ionic composition of hagfish extracellular fluids were similar to that of seawater (SW), and as a result, hagfish have been touted as the model craniate osmo- and ionoconformer for nearly 80 years. What has been largely overlooked when considering this model, however, is the lack of direct symmetry between the ionic concentrations of SW and hagfish plasma, particularly with respect to divalent cations such as Ca^{2+} and Mg^{2+} where plasma concentrations are approximately half that of SW (Robertson 1954, 1976; Smith 1932). There are also subtle variations in the concentrations of Na^+ and Cl^- between hagfish plasma and water (Robertson 1954, 1976; Smith 1932), although it is unknown if the lack of symmetry indicates some form of active regulation.

Communicated by I. D. Hume.

B. A. Sardella (✉)
Department of Biology, Eastern Washington University, Cheney, WA 99004, USA
e-mail: bsardella@ewu.edu

D. W. Baker · C. J. Brauner
Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, BC V6S 1Z4, Canada

Based on dual observations of a reversible decrease of net acid excretion when acclimated to Na^+-free water, and a reversible decrease of net base excretion when acclimated to Cl^--free water, Evans (1984) suggested that hagfish possess Na^+/H^+ (NHE) and Cl^-/HCO_3^- (AE) exchangers on the branchial epithelium that functioned in parallel for acid–base regulation. Given that hagfish are thought never to have invaded freshwater (FW), Evans (1984) further speculated that within chordates, the ability to exchange acid/base equivalents with counter-ions evolved in SW, and thus was a 'pre-adaptation' (exaptation) for the invasion of FW. Later it was supported by Edwards et al. (2001) that hagfish gills may play a role in acid–base regulation, where NHE expression was found in Atlantic hagfish (*Myxine glutinosa*) gill tissue following acid infusion. Further support was added when mitochondrion-rich cells (MRCs), which are the key cell type for acid–base balance and ionoregulation in more derived species, were characterized in hagfish gills by Mallat et al. (1987). They also noted that hagfish MRCs did not share the paracellular junctions that play a critical role in Na^+ excretion in marine ionoregulators (Marshall 2002), indicating that these cells likely had little, if any, ionoregulatory role. In the last 30 years, many enzymes associated with ionoregulatory functions, such as Na^+, K^+-ATPase (NKA), carbonic anhydrase (CA) (Mallat et al. 1987), and immunolocalized V-type H^+-ATPase (VHA) (Tresguerres et al. 2006), have been localized to the MRCs of hagfish and the roles of these enzymes continue to be investigated.

The purpose of our experiment was to gain more insight into the osmo- and ionoregulatory capacity of hagfish following perturbations in the ionic composition of their environment. Towards the end, we altered both salinity (24, 32, 40, and 48 g/l) and ionic composition (replacement of Na^+ with Ca^{2+} or Mg^{2+} at constant salinity) and characterized the changes in plasma ionic status, pH, and total carbon dioxide (TCO_2). Because hagfish have been assumed to be ionoconformers, investigations of their ability to handle salinity challenges have been few (Cholette et al. 1970; Toop and Evans 1993). Since past evidence has shown that hagfish likely have a regulatory capacity for Mg^{2+} and Ca^{2+}, we sought to place additional challenges on their ability in the hope of further elucidating their mechanisms.

Materials and methods

Pacific hagfish (*Eptatretus stoutii*) were captured using baited traps at a depth of approximately 100 m in Barkley Sound and transported to the Bamfield Marine Science Centre, Bamfield, British Columbia, Canada, where animals were held and experiments were conducted in accordance with BSMC Animal Care Committee protocol. Animals were held in dark flow-through SW tanks (12°C) for 1–2 weeks prior to experiments and weighed 103 ± 17.3 g at the time of salinity transfer. Dead fish were placed in the tanks to provide food, although there was no evidence of feeding, and no food was made available during the exposure to experimental conditions.

Experiment 1: The effect of 48 h exposure to altered salinity on osmo- and ionoregulatory status

We acclimated hagfish to 24, 32 (SW), 40, and 48 g/l salinity for 48 h ($n = 8$). Salinity was decreased from full strength SW to 24 g/l by dilution of Barkley Sound water with well water, and salinities of 40 and 48 g/l were produced by addition of Instant Ocean synthetic sea salts (Spectrum Brands Inc., Atlanta, GA). Experimental tanks were 50 l in volume, and were kept aerated and dark. The exposures were static, so in order to keep ammonia levels minimal, 50% of tank volume was replaced after 24 h, feeding was withheld, and AmmoLock (API, Chalfont, PA) was added to the water as a precautionary measure in case ammonia began to accumulate. After 48 h, hagfish were removed from their respective tanks, given a lethal dose of buffered MS-222 diluted to a final concentration of 1.0 g/l and then terminally sampled (see below).

Experiment 2: The effect of elevated Mg^{2+} and Ca^{2+} at constant salinity on osmo- and ionoregulatory status

Hagfish were given a 6 days exposure to water with a portion of the $[Na^+]$ substituted by either Mg^{2+} or Ca^{2+} ($n = 8$). Water was prepared by first diluting 32 g/l SW to 24 g/l total salinity with well water as described above, and then adding $MgCl_2$ (74.0 mM) or $CaCl_2$ (40.7 mM) to increase salinity back to 32 g/l. The result was 32 g/l salinity water with a twofold increase of $[Mg^{2+}]$ and a sixfold increase of $[Ca^{2+}]$. Hagfish were sampled 2 and 6 days following transfer from SW to the respective media.

Sampling protocol

Each hagfish was weighed prior to- and following exposure to experimental treatments. Following anesthetization, blood was withdrawn from their large caudal sinus into a 1 ml syringe that had been previously rinsed with Li-Heparin (1.76 mg/ml, Sigma). Blood was placed into a heparinized microcentrifuge tube, blood pH (pHe) was measured using a thermostated capillary pH electrode (BMS 3 Mk 2) and pH meter (PHM 84; Radiometer, Westlake, OH), and the remaining blood was centrifuged for 3 min. Plasma TCO_2 was measured using a CO_2 analyzer (965, Corning, Corning, NY) and plasma osmolality was measured using a Wescor 5500 Vapor Pressure Osmometer (Wescor, Logan, UT). Plasma $[Na^+]$, $[Mg^{2+}]$, and $[Ca^{2+}]$ was measured on

diluted samples using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA) and plasma [Cl^-] was measured using an HBI digital chloridometer. A section of dorsal epaxial muscle approximately 1.0 g in weight was removed from the left 'side' of the hagfish, rinsed, patted dry, and desiccated on a pre-weighed piece of aluminum foil at 70°C for 96 h, by which time, there was no further change in weight of the dried muscle. Muscle water content was measured as the percent weight change following desiccation.

Statistics

The effects of all treatments were analyzed using a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey HSD test when results were significant ($\alpha = 0.05$). In addition, linear regression analysis was conducted to investigate the correlation between plasma and water osmolality. All data were analyzed for normality and equal variance prior to ANOVA. All ANOVAs were conducted using SigmaStat version 3.0 and regressions were conducted using SigmaPlot version 9.0.

Results

No mortality was observed during any of our exposures. During the majority of observations, hagfish remained coiled and quiescent on the bottom of the experimental tanks, however, at 24 g/l they appeared slightly bloated and were less likely to be completely coiled. A comparison of the dominant ionic concentrations between SW and plasma from control hagfish is presented in Table 1.

Experiment 1: The effect of altered salinity on osmo- and ionoregulatory status

Plasma osmolality was dramatically affected after 48 h of exposure to the various salinities (Fig. 1a; $P < 0.001$), and was highly correlated with water osmolality (Fig. 1b; $P < 0.0001$, $m = 0.966$, $r^2 = 0.996$). Plasma [Na^+] and [Cl^-] was either increased or decreased in the hyper- and hypo-saline groups, respectively (Fig. 2a; $P < 0.001$), whereas plasma [Mg^{2+}] was only significantly different in the 48 g/l-exposed hagfish and was significantly elevated relative to all other salinities (Fig. 2b). Variation of ambient salinity significantly affected both hagfish weight (Fig. 3a; $P < 0.001$) and muscle water content (Fig. 3b; $P < 0.001$); both were inversely proportional to changes in water salinity.

Table 1 Ionic concentrations of seawater (32 g/l) and hagfish plasma

Ion	[Water]	[Plasma]
Na^+	450.0	506.0 ± 6.0
Cl^-	478.0	466.5 ± 5.3
Mg^{2+}	35.6	14.3 ± 0.49
Ca^{2+}	7.0	3.06 ± 0.07
TCO_2	1.8	4.49 ± 0.91
Osmolality	875.0	937 ± 2.52

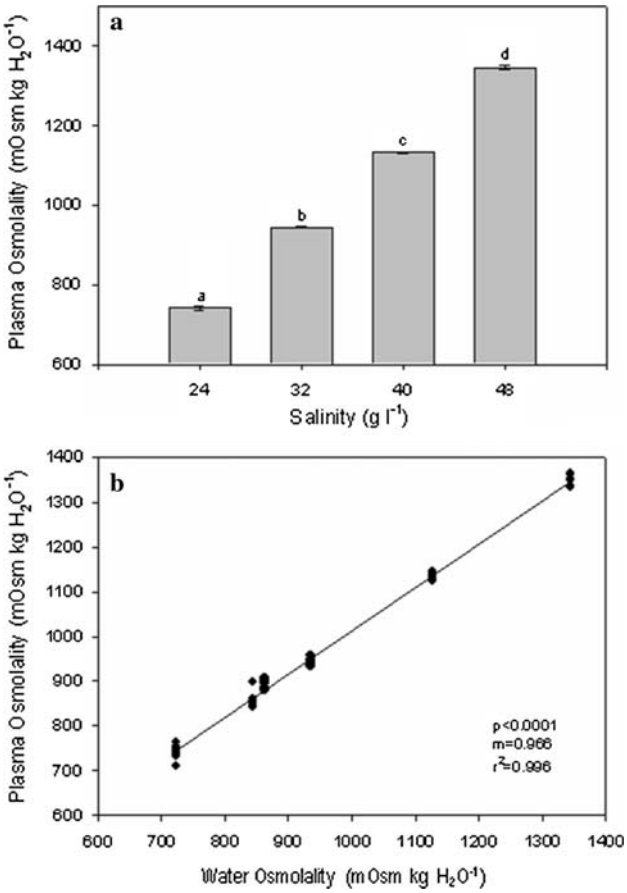

Fig. 1 **a** The effect of ambient salinity on the plasma osmolality of Pacific hagfish. Letters indicate a significant difference in mean values between salinities measured by one-way-ANOVA ($P < 0.001$, $n = 8$). **b** Linear regression plot between water and plasma osmolality. Figure includes individual values from salinity treatments as well as manipulations of [Ca^{2+}] and [Mg^{2+}] ($P < 0.0001$, $m = 0.966$, $r^2 = 0.996$)

Experiment 2: The effect of elevated Mg^{2+} and Ca^{2+} at constant salinity on osmo and ionoregulatory status

The water osmolality of the Mg^{2+} and Ca^{2+} replacement treatments was approximately 10% lower than that of straight SW (Table 2) and hagfish plasma osmolality decreased proportionally (Table 2). Interestingly, plasma osmolality was about 5% higher, relative to water osmolality in the high Mg^{2+} and Ca^{2+} treatments, as well as in SW (Tables 1, 2). The substitution of Mg^{2+} for Na^+ increased water [Mg^{2+}] from 35 to 74 mM, yet remarkably, plasma

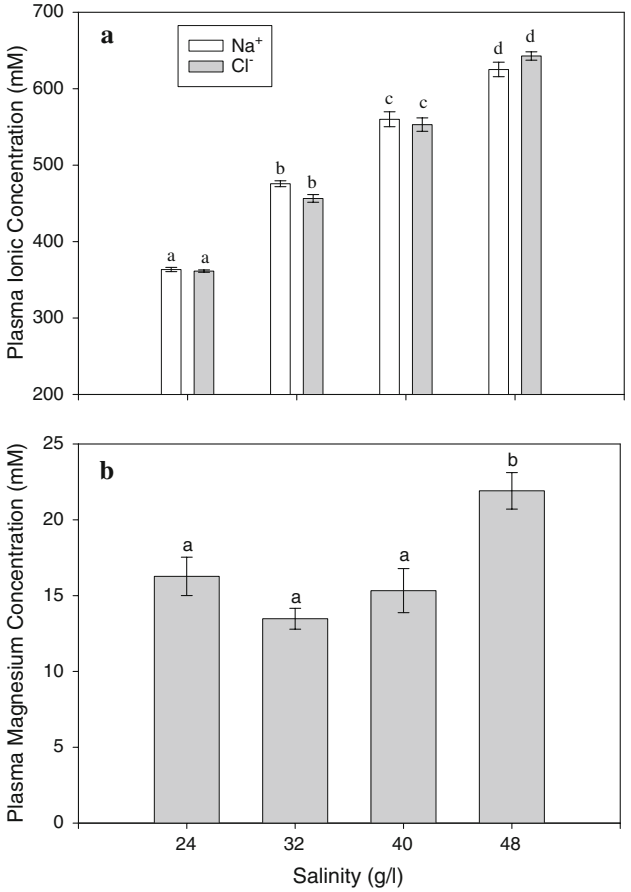

Fig. 2 The effect of ambient salinity on hagfish **a** plasma [Na^+] and [Cl^-], and **b** plasma [Mg^{2+}] ($P < 0.001$, $n = 8$)

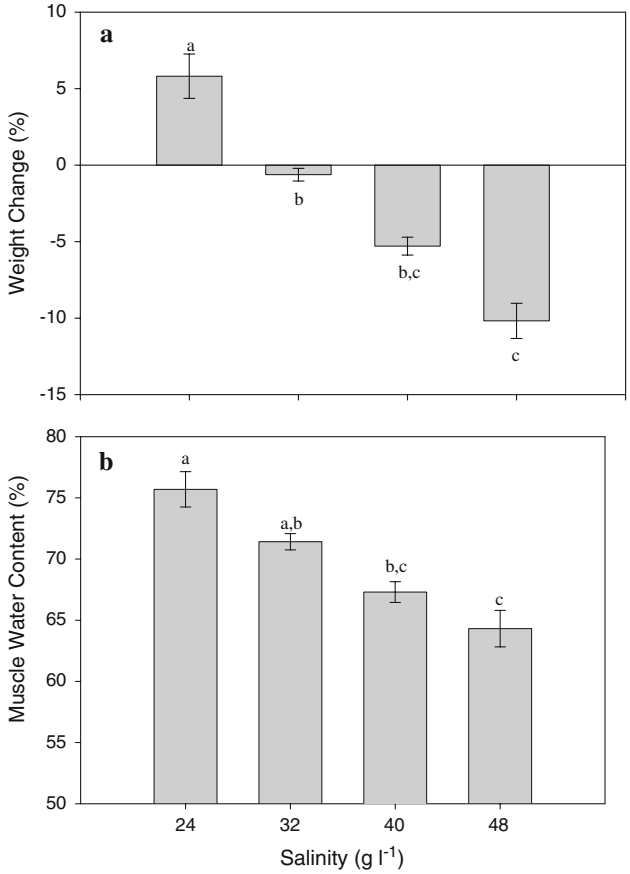

Fig. 3 The effect of ambient salinity on **a** whole animal weight and **b** muscle water content ($P < 0.001$, $n = 8$)

[Mg^{2+}] did not increase and was even slightly decreased after 2 days of exposure ($P = 0.03$) before returning to control levels at 6 days (Table 2). There was no change in plasma [Ca^{2+}] throughout the exposure (Table 2). Plasma [Na^+] (429 ± 6.8 mM) and [Cl^-] (403 ± 5.6 mM) were significantly lower in both Mg^{2+} and Ca^{2+} replacement treatments relative to hagfish that were held in SW, but both groups had similar $[Ion]_{out}/[Ion]_{in}$ ratios with respect to both [Na^+] and [Cl^-]. Finally, in hagfish exposed to elevated [Mg^{2+}], plasma pH and TCO_2 were significantly increased after 6 days (Fig. 4a, b, respectively; both $P < 0.001$).

In the Ca^{2+} replacement experiment, water [Ca^{2+}] was increased almost sixfold, from 7 to 40 mM (>500%), however the significant increase that we observed at 6 days was still far below that of the water (43%; Table 2). Once again, water osmolality, [Na^+], and [Cl^-] were decreased relative to SW, and plasma osmolality was decreased relative to SW-exposed animals (Table 2). The ratios of [Na^+] and [Cl^-] between plasma and water were also maintained in similar fashion to hagfish in SW, despite the water having a 25% lower [Na^+]. Curiously, plasma [Mg^{2+}] was signifi-

Table 2 Ionic concentrations of water at 32 g/l total dissolved solutes with Mg^{2+} or Ca^{2+} replacement of Na^+, as well as of hagfish plasma at 2 and 6 days

Treatment	Ion	[Water]	[Plasma]	
			2 days	6 days
Mg	Na^+	390.0	425 ± 7.4*	439 ± 6.8*
	Cl^-	463.0	410 ± 6.5*	404 ± 5.7*
	Mg^{2+}	74.0	11.9 ± 0.75*	13.6 ± 0.41
	Ca^{2+}	6.5	2.99 ± 0.14	2.66 ± 0.15
	TCO_2	1.8	6.45 ± 1.4	$12.82 \pm 0.52^{\dagger}$
	Osmolality	761.0	811 ± 6.2*	791 ± 8.0*
Ca	Na^+	390.0	425 ± 6.5*	411 ± 6.2*
	Cl^-	442.0	386 ± 5.3*	403 ± 3.5*
	Mg^{2+}	38.7	11.0 ± 0.79*	9.04 ± 0.44*
	Ca^{2+}	40.7	3.43 ± 0.18	$4.48 \pm 0.31^{\dagger}$
	TCO_2	1.8	$12.7 \pm 1.1^{\dagger}$	$8.48 \pm 0.80^{\dagger\dagger}$
	Osmolality	792.0	785 ± 4.4*	780 ± 4.52*

* Significantly decreased from SW group. † Significantly increased from SW group. †† Significantly increased form SW group but decreased from 2 days

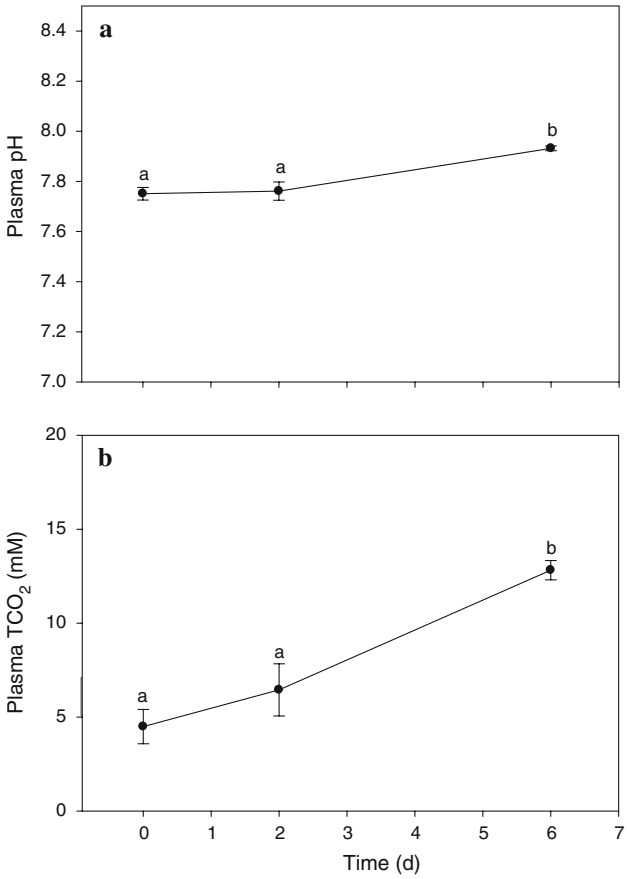

Fig. 4 Changes in **a** plasma pH and **b** plasma total CO_2 in hagfish exposed to SW with reduced [Na^+] and elevated [Mg^{2+}]. Letters indicate a significant difference in mean values between salinities measured by one-way-ANOVA ($P < 0.001$, $n = 8$)

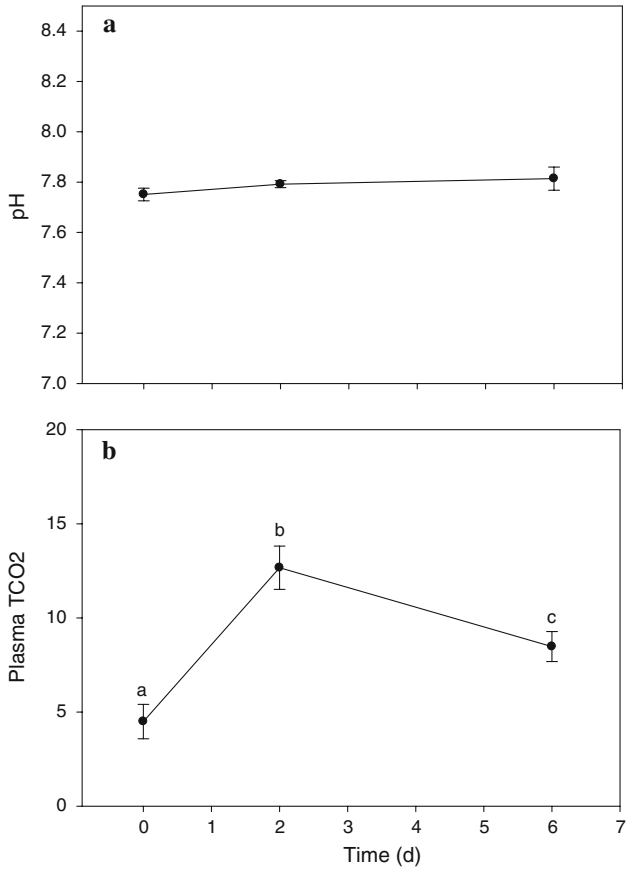

Fig. 5 Changes in **a** plasma pH and **b** plasma total CO_2 in hagfish exposed to SW with reduced [Na^+] and elevated [Ca^{2+}] ($P < 0.001$, $n = 8$)

cantly decreased at 2 and 6 days following transfer, even though water [Mg^{2+}] was unchanged from SW level. Finally, there was no change in plasma pH over the 6 days period (Fig. 5a), but TCO_2 was significantly elevated at both 2 and 6 days (Fig. 5b; $P < 0.001$).

Discussion

Hagfish have long been described as marine osmo- and ionoconformers based upon the similarity of ionic composition between their plasma and SW (Wright 2007). There have been few studies where environmental salinity or ionic composition was manipulated in order to determine if hagfish had any control of plasma ionic concentrations (Cholette et al. 1970; McFarrland and Munz 1965; Toop and Evans 1993). In the present study, the response to salinity manipulation, either through dilution of SW or addition of artificial sea salts, indicated that hagfish had no capacity to regulate plasma osmolality, [Na^+], or [Cl^-] (Figs. 1, 2a), but they were effective at maintaining plasma [Mg^{2+}] (Fig. 2b). Furthermore, when water [Mg^{2+}] or [Ca^{2+}] were elevated at a constant salinity, plasma [Mg^{2+}] and [Ca^{2+}] remained virtually unchanged (Table 2). Although these environmental conditions may not be experienced by hagfish in the ocean, this study clearly demonstrates that hagfish have greater control over their extracellular environment than previously thought. A great deal remains to be learned about the physiology of this basal craniate, as well as what the implications for the evolution of ion and acid–base regulation may be.

Experiment 1: The effect of altered salinity on osmo- and ionoregulatory status

Plasma osmolality, [Na^+], and [Cl^-] remained similar to water values when hagfish were exposed to 24, 32, 40, or 48 g/l salinity (Figs. 1, 2a). Hagfish plasma osmolality was tightly correlated with that of the water (Fig. 1b; $r^2 = 0.996$), indicating a clear lack of osmotic control by these animals. The results were a 23% decrease of hagfish plasma [Na^+] and [Cl^-] after 48 h of exposure to 24 g/l salinity, and 17 and 29% increases of plasma [Na^+] and [Cl^-] when exposed to 40 and 48 g/l, respectively. The

nearly identical changes of plasma ionic concentrations with environmental salinity clearly indicate a lack of capacity to regulate either of these ions, which is consistent with the conclusions of McFarrland and Munz (1965) and Smith (1932). We did not observe the same pattern for plasma [Mg^{2+}] (Fig. 2b), which did not change relative to SW values following 2 days of exposure to 24 or 40 g/l. The maintenance of plasma [Mg^{2+}] at 24 and 40 g/l may indicate that these animals exerted control over their internal milieu, but clearly not with respect to extracellular [Na^+] and [Cl^-]. The possibility of divalent cation regulation is very intriguing and potential mechanisms are discussed in greater detail in the following section. When exposed to 48 g/l salinity, hagfish plasma [Mg^{2+}] increased by 69%, which was much higher than what would be predicted based upon changes of plasma [Na^+] and [Cl^-] (29%). An increase of such magnitude likely indicates that any potential regulatory mechanisms were either hindered by the high-salinity exposure (Hochachka and Somero 2002), and that the hagfish may have succumbed had the duration of exposure been longer.

Varying ambient salinity also affected hagfish weight as early as 24 h (data not shown), and this change appeared to be complete by 48 h (Fig. 3a). Similarly, the muscle water content was also significantly affected (Fig. 3b). The muscle compartment represents a large proportion of total body mass, so not surprisingly, changes in muscle water content were consistent with changes in total body weight as salinity was adjusted. In a previous study, hagfish exposed to 80% SW were able to recover from water weight gain, reducing weight to control levels by 7 days, but they were not able to recover from the weight loss associated with an elevation in salinity to 122% SW (McFarrland and Munz 1965). Bellamy and Jones (1961) characterized the intracellular composition of hagfish muscle and found it to be approximately 40% lower in inorganic ion concentration relative to plasma (Na^+, K^+, and Cl^-), but found that organic osmolyte level (Amino Acids, TMAO) that exceeded 200 mM (Bellamy and Jones 1961). The capability of hagfish to regulate compatible osmolytes under dehydration or dilution of the extracellular space is still unknown, but our data indicate that 2 days of exposure to hypo- or hyperosmotic conditions was insufficient for a recovery response (Fig. 3).

Experiment 2: The effect of elevated [Mg^{2+}] and [Ca^{2+}] at constant salinity on osmo- and ionoregulatory status

The replacement of water Na^+ with either Mg^{2+} or Ca^{2+} at constant salinity resulted in a doubling of water [Mg^{2+}] and a sixfold increase in water [Ca^{2+}]. Remarkably, there was no significant change in plasma [Mg^{2+}] and a significant change in plasma [Ca^{2+}] where concentrations were still far below that of the water, which may indicate that hagfish have a tremendous capacity to regulate these plasma ions. It is possible that unlike Na^+ and Cl^-, neither Mg^{2+} nor Ca^{2+} equilibrated between water and hagfish plasma at 2 or 6 days due to either low-epithelial permeability or insufficient time; however, based upon data from experiment 1, we do not feel this was the case. When hagfish were exposed to an increase (to 40 g/l) or decrease (to 24 g/l) in environmental salinity, there was a rapid equilibration of plasma osmolality, [Na^+] and [Cl^-], as well as rapid changes in whole animal weight. If the changes were due to water movement alone and no ion regulatory ability existed, exposure to 40 g/l would be expected to result in a 25% increase in all plasma ions (including Mg^{2+}) due to contraction of plasma volume, and exposure to 24 g/l would be expected to result in a 25% decrease in all plasma ions due to an expansion of the plasma volume. Because plasma [Mg^{2+}] does not change during exposure to 40 or 24 g/l, there must be a significant elimination and uptake, respectively, of [Mg^{2+}] during exposure to these salinities, although direct evidence is still lacking.

It has been previously noted that hagfish plasma [Mg^{2+}] and [Ca^{2+}] were approximately half that of SW (Cholette et al. 1970; Robertson 1954, 1976), and we have noted a similar relationship here (Table 1). If plasma [Mg^{2+}] and [Ca^{2+}] were to have increased during the salinity manipulations proportionally to environmental levels, as was observed with [Na^+] and [Cl^-] in experiment 1, we would then predict a plasma [Mg^{2+}] value of approximately 37.0 mM (actual [Mg^{2+}] = 13.6 ± 0.41 mM) in the Mg^{2+} substitution experiment, and a plasma [Ca^{2+}] value of 20.4 mM (actual [Ca^{2+}] = 4.48 ± 0.03 mM) in the Ca^{2+} substitution experiment. Previous studies, as well as ours, have shown slight offsets between plasma and water with respect to [Na^+] and [Cl^-], where plasma [Na^+] was slightly increased relative to the water, and plasma [Cl^-] was slightly decreased relative to the water (Table 1; McFarrland and Munz 1965; Smith 1932). This may be associated with regulating plasma Mg^{2+} and Ca^{2+} at levels much lower than the environment where charge balance would necessitate an increase in other cations (Na^+) or decrease in anions (Cl^-).

The mechanism through which plasma [Mg^{2+}] and [Ca^{2+}] were regulated is unknown, however, the capacity for this at the level of the hagfish kidney has been shown to be minimal (Alt et al. 1981). Interestingly, it has been previously shown that the concentrations of these ions are anywhere from 8–10 times higher in hagfish bile relative to plasma, indicating a potential mechanism for removing Mg^{2+} and Ca^{2+} from circulation (Forster and Fenwick 1994; Robertson 1976). One potential mechanism, has yet to be explored, is associated with epithelial HCO_3^- secretion that is crucial in the intestine of marine teleosts (Wilson et al. 2002), which may maintain internal concentrations by

preventing their initial absorption. In this model, Mg^{2+} and Ca^{2+} form precipitates with the secreted HCO_3^-, precluding absorption across the intestinal epithelium. A consequence of HCO_3^- secretion into the intestine is an associated blood acidosis, as the HCO_3^- is derived by the hydration of CO_2 in the presence of carbonic anhydrase (Wilson and Grosell 2003). The excess protons must be compensated by either excretion of acid equivalents in exchange for Na^+ and/or uptake of base equivalents in exchange for Cl^- by gill NHE and AE, respectively. The movements of these counter-ions would be consistent with the differences in hagfish plasma [Na^+] and [Cl^-] relative to the environment (Table 1; Bellamy and Jones 1961; Smith 1932). The acidosis resulting from increased HCO_3^- secretion during exposure to elevated water [Mg^{2+}] and [Ca^{2+}] may be responsible for the dramatic increase in TCO_2 (and thus HCO_3^-) of the blood required to maintain plasma pH (Figs. 4, 5). An increase of acid–base exchanges also explains why the variation of counter-ion concentrations deviated to such a large degree from environmental values in response to elevated water [Mg^{2+}] and [Ca^{2+}]. As a result, plasma [Na^+] was 20–30 mM higher, and plasma [Cl^-] 40–60 mM lower than the environment during elevated Mg^{2+} and Ca^{2+} treatments, respectively (Table 2).

The potential for epithelial HCO_3^- secretion in these animals is intriguing from an evolutionary perspective, as hagfish represent the most pleisiomorphic extant craniate and are thought to have ancestry that never entered FW (Janvier 2001). In marine fish, HCO_3^- secretion across the intestinal epithelium is thought to serve two critical roles. First, the formation of precipitates with Mg^{2+} and Ca^{2+} prevents them from being absorbed into the blood, thus maintaining plasma levels low relative to the SW environment, as discussed above. Second, precipitate formation reduces the osmolality of the luminal fluid, which favors the osmotic absorption of water across the epithelium that is crucial to marine osmo- and ionoregulators that constantly lose water to the environment (Wilson et al. 1996, 2002; Wilson and Grosell 2003). Evidence of this mechanism in hagfish is intriguing, as it indicates that epithelial secretion of HCO_3^- potentially evolved prior to the craniate invasion of FW and before the evolution of plasma compartment ionoregulation. We speculate that the secretion of HCO_3^- evolved to maintain plasma [Mg^{2+}] and [Ca^{2+}] below environmental levels (both of which negatively affect nervous function and muscle contraction if elevated) but may have been an exaptation for the development of water-absorption mechanisms in the intestine of marine osmoregulators. Our data further indicate that the Ca^{2+} was not as well-maintained as magnesium, evident by the slight rise in Ca^{2+} concentration and the much greater change in TCO_2 following Ca^{2+} exposure. Future studies should focus on the gill and intestine of these animals, as the secretion abilities of the hagfish kidney with respect to Mg^{2+} and Ca^{2+} have been shown to be minimal (Alt et al. 1981). The characterization of the mechanism for Mg^{2+} and Ca^{2+} regulation in hagfish, and its localization to a particular tissue, will be a crucial focus of future studies and has dramatic potential for discovery.

Acknowledgments We would like to give special thanks to those who assisted in the execution of this research, including Dr. Greg Goss and Scott Parks of the University of Alberta, and Dr. Jonathan Wilson of CIIMAR, Portugal. We also would like to thank Bruce Cameron for invaluable assistance at BMSC during these experiments. This research was supported by an NSERC Discovery grant to CJB.

References

Alt JM, Stolte H, Eisenbach GM, Walvig F (1981) Renal electrolyte and fluid excretion in the Atlantic hagfish *Myxine glutinosa*. J Exp Biol 91:323–330

Bellamy D, Jones IC (1961) Studies on *Myxine glutinosa*. I. The chemical composition of the tissues. Comp Biochem Physiol 3:175–183

Cholette C, Gagnon A, Germian P (1970) Isosmotic adaption in *Myxine glutinosa* L.Variations of some parameters and role of the amino acid pool of the muscle cells. Comp Biochem Physiol 33:333–346

Edwards SL, Claiborne JB, Morrison-Shetlar AI, Toop T (2001) Expression of Na^+/H^+ exchanger mRNA in the gills of the Atlantic hagfish (*Myxine glutinosa*) in response to metabolic acidosis. Comp Biochem Physiol 130A:81–91

Evans DH (1984) Gill Na^+/H^+ and Cl^-/HCO_3^- exchange systems evolved before the vertebrates entered freshwater. J Exp Biol 113:465–469

Forster ME, Fenwick JC (1994) Stimulation of calcium efflux from the hagfish (*Eptatretus cirrhatus*), gill pouch by an extract of corpuscles of Stannius from an eel (*Anguilla dieffenbachii*): Teleostei. Gen Comp Endocrinol 94:92–103

Hochachka PW, Somero GN (2002) Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, New York

Janvier P (2001) Living primitive fishes and fishes from deep time. In: McKenzie DJ, Brauner CJ, Farrell AP (eds) Fish physiology. Elsevier, San Diego, CA, p 51

Mallat J, Conley DM, Ridgway RL (1987) Why do hagfish have gill "chloride cells" when they need not regulate plasma NaCl concentration. Can J Zool 65:1956–1965

Marshall WS (2002) Na^+, Cl^-, Ca^{2+}, and Zn^{2+} transport by fish gills: a retrospective review and prospective synthesis. J Exp Zool 293:264–283

McFarrland WN, Munz FW (1965) Regulation of body weight and serum composition by hagfish in various media. Comp Biochem Physiol 17:383–398

Robertson JD (1954) The chemical composition of the blood of some aquatic chordates, including members of the Tunicata, Cyclostomata and Osteichthyes. J Exp Biol 31:424–442

Robertson JD (1976) Chemical composition of the body fluids and muscle of the hagfish *Myxine glutinosa* and the rabbit-fish *Chimaera monstrosa*. J Zool 178:261–277

Smith HW (1932) Water regulation and its evolution in fishes. Quart Rev Biol 7:1–26

Toop T, Evans DH (1993) Whole animal volume regulation in the Atlantic hagfish, (*Myxine glutinosa*), exposed to 85% and 115% sea water. Bull Mt Des Isl Biol Lab 32:98–99

Tresguerres M, Parks SK, Goss GG (2006) V-H^+-ATPase, Na^+, K^+-ATPase and NHE2 immunoreactivity in the gill epithelium of

the Pacific hagfish (*Epatretus stoutii*). Comp Biochem Physiol 145:312–321

Wilson RW, Grosell M (2003) Intestinal bicarbonate secretion in marine teleost fish- source of bicarbonate, pH sensitivity, and consequences for whole animal acid–base and calcium homeostasis. Biochim Biophys 1618:163–174

Wilson R, Gilmour K, Henry R, Wood C (1996) Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid–base balance? J Exp Biol 199:2331–2343

Wilson RW, Wilson JM, Grosell M (2002) Intestinal bicarbonate secretion by marine teleost fish—why and how? Biochim Biophys 1566:182–193

Wright PA (2007) Ionic, osmotic and nitrogenous waste excretion. In: McKenzie DJ, Farrell AP, Brauner CJ (eds) Fish physiology. Elsevier, New York, pp 283–319