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Physiological Effects of High Salinity in Teleost Fish

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Natural exposure to hypersaline environments occurs in many fish species (*e.g.* in tidal pools, lakes, estuaries, or due to migration). Fish in environments that are more concentrated than their extracellular fluids experience continual diffusive water loss which they combat by drinking. Intestinal Na^+ and Cl^- absorption drives water uptake and concentrates impermeable $MgSO_4$ in the lumen. Epithelial Cl^- absorption occurs in part by apical Cl^-/HCO_3^- exchange, with HCO_3^- provided by transepithelial transport and/or by carbonic anhydrase-mediated hydration of endogenous CO_2. Hydration of CO_2 also liberates H^+, which is excreted across either the basolateral or apical membranes. Transport of water, ions, and acid-base equivalents are therefore intricately linked in the response of teleosts to high salinity. This dissertation compares three teleost species of varying salinity tolerance: the freshwater, anadromous rainbow trout (*Oncorhynchus mykiss*), the marine gulf toadfish (*Opsanus beta*), and the highly euryhaline killifish (*Fundulus heteroclitus*). In toadfish, intestinal HCO_3^- secretion increases with salinity, but the resulting acid-base disturbance is compensated by branchial net acid excretion. Toadfish exposed to hypersaline waters containing artificially low [$MgSO_4$] have enhanced survival and osmoregulation compared to fish in water of natural ionic composition. Upper salinity tolerance is determined by intestinal capacity for Na^+, Cl^-, and water absorption, which is limited by luminal [$MgSO_4$], rather than renal or branchial processes. Rainbow trout exposed to 70% seawater adjust from

hyper- to hypoosmoregulatory strategies within 24-48 hours. Ion transporters important to both intestinal osmoregulation and maintenance of acid-base balance (NHE1, NHE2, SLC26a6) in the trout differed from other teleost species in their contribution to intestinal osmoregulation. Methods utilizing isolated killifish intestinal tissue under physiologically relevant conditions were discovered to be ineffectual. Regardless, the extremely salinity-tolerant killifish was shown to require relatively few physiological changes following acute transfer from 35 to 70 ppt seawater, and epithelial permeability adjustments occur within 24 h post-transfer. This dissertation examines the contribution of intestinal transport of water, ions, and acid-base equivalents to the osmoregulation of fish in hypersaline environments. Comparisons among species exposed to their upper salinity tolerance range expand our understanding of the essential iono- and osmoregulatory requirements of teleost fish.

Publications Note

The publication status of each research chapter in this dissertation is as follows:

Chapter 2 (Genz et al., 2008)

Chapter 3 (In Review, American Journal of Physiology: Regulatory, Integrative and Comparative Physiology)

Chapter 4 (In Preparation)

Chapter 5 (In Preparation)

Within the time frame of this dissertation, additional contributions by J. Genz were published, as follows:

Grosell,M., **Genz,J**., 2006. Oubain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. American Journal of Physiology 291(4), R1145-R1156.

Grosell,M., **Genz,J.**, Taylor,J.R., Perry,S.F., Gilmour,K.M., 2009a. The involvement of H+-ATPase and carbonic anhydrase in intestinal HCO3- secretion in seawater-acclimated rainbow trout. Journal of Experimental Biology 212(12), 1940-1948.

Perry, S.F., Braun, M.H., **Genz, J.**, Vulesevic, B., Taylor, J.R., Grosell, M., & Gilmour, K.M. 2010. Acid-base regulation in the plainfin midshipman (*Porichthys notatus*), an aglomerular marine teleost. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 180:1213-1225.

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Chapter 1

Introduction

Marine teleosts regulate the osmotic pressure of their extracellular fluids at ~300-350 mOsm, approximately 2/3 lower than seawater (Shehadeh and Gordon, 1969). Therefore, fish that have an internal milieu hypoosmotic to their surroundings experience continual water loss and influx of salts. This diffusive water loss is opposed by drinking the ambient water. Water and salt (particularly Na^+ and Cl^-) are actively absorbed by the gastrointestinal tract, modifying the ionic composition of the ingested fluid (Smith, 1930; Skadhauge, 1969; Skadhauge, 1974; Ando, 1975). Branchial excretion of monovalent ions and rectal or renal excretion of divalent ions (Beyenbach, 2004; Marshall and Grosell, 2005; Evans et al., 2005) results in neutral net water balance. Osmoregulation is thus an integrative process relying primarily on the gut, gill, and kidney.

Apical Cl^-/HCO_3^- exchange accounts for a substantial portion of intestinal Cl^- absorption occurring independently of Na^+ absorption (Grosell et al., 2001; Grosell, 2006). In all fish examined to date, the secreted HCO_3^- is produced either by carbonic anhydrase-mediated hydration of endogenous metabolic CO_2 (Wilson and Grosell, 2003; Grosell et al., 2005; Grosell and Genz, 2006) and/or extracellular transport across the basolateral membrane by Na^+/HCO_3^- cotransport (Ando and Subramanyam, 1990; Taylor et al., 2010). The amount of apically secreted HCO_3^- derived from each of these two sources varies among species (Grosell, 2006). In the gulf toadfish (*Opsanus beta*), each contributes approximately 50% of intestinally secreted HCO_3^- (Grosell and Genz, 2006); in the rainbow trout (*Oncorhynchus mykiss*) all the HCO_3^- can be accounted for by intracellular CO_2 hydration (Grosell et al., 2009a).

Osmoregulation of teleosts inhabiting both marine and freshwater environments has been studied extensively and is fairly well understood, but investigations of fish exposed to hypersaline environments are relatively rare. The available literature indicates, in general, that exposure to hypersalinity increases characteristic traits related to osmoregulation exhibited by fish in seawater (*e.g.* increased drinking rate, plasma osmotic pressure, intestinal absorption and plasma concentrations of Na^+ and Cl^-, Na^+-K^+-ATPase activity in the gut and gill).

Fish exposed to high salinity also increase HCO_3^- secretion into the intestinal lumen to facilitate water absorption (McDonald and Grosell, 2006; Grosell, 2006). Active intestinal secretion of base equivalents has implications for maintenance of whole-animal acid-base balance. The relatively alkaline blood pH (~7.7 to 8.1) maintained by fish is primarily dependent on branchial transport of acid-base molecules (Marshall and Grosell, 2005; Evans et al., 2005). In seawater fish, H^+ excretion from the gill is accomplished via Na^+/H^+ exchange (NHE) and V-type H^+-ATPase, while base excretion is predominantly via apical Cl^-/HCO_3^- exchange (Claiborne et al., 1997; Claiborne et al., 2002).

As discussed above, intracellular CO_2 hydration in the intestine results in production of HCO_3^-, which is transported across the apical membrane. This reaction also produces H^+, which is transported across the basolateral membrane into the extracellular fluids (Grosell et al., 2001). In *O. beta*, this occurs by an NHE-like transport process (Grosell and Genz, 2006) and results in net acid absorption (Grosell and Taylor, 2007) which balances the HCO_3^- secretion. Increased intestinal HCO_3^- secretion due to increased need for intestinal fluid absorption, therefore, may result in systemic

acid gain and possible metabolic acidosis. Increased intestinal base secretion rates associated with osmoregulation in hypersalinity would therefore be expected to have an acid-base effect. Chapter 2 focuses on the impact of intestinal HCO_3^- secretion on whole animal acid-base balance in toadfish acclimated to 9, 35, and 50 ppt salinities.

Building from the findings of Chapter 2, specifically estimated absorption rates of Na^+, Cl^-, and water occurring in toadfish exposed to 50 ppt seawater, Chapter 3 takes an in-depth look at the processes possibly limiting water absorption, and therefore survival, in toadfish exposed to hypersalinity, focusing on the concentration of impermeable divalent ions in the intestinal lumen. Salt and water transport by toadfish *in vivo* and by isolated intestinal epithelia were quantified to determine the limiting factors for marine fish to tolerate elevated salinity.

Each of the three organs responsible for osmoregulation by teleost fish may be limiting for survival in high salinity. First, the gill may be limiting with regards to more difficulty excreting Na^+ and Cl^- against increased uphill gradients in elevated ambient salinity. Secondly, three impacts on the intestinal contribution to osmoregulation during hypersaline exposure are likely important in determining tolerance: an increase in drinking rate, increased Na^+ and Cl^- absorption by the epithelium (Grosell et al., 2009a), and higher concentrations of Mg^{2+} and SO_4^{2-} in the intestinal lumen due to preferential absorption of Na^+, Cl^-, and water (Chapter 2). Third, the kidney may potentially limit survival in hypersaline environments due to the increased demand for renal $MgSO_4$ excretion. This is likely to result in higher rates of urine flow (and thus water loss) since marine teleosts are incapable of forming hyperosmotic urine (Hickman and Trump, 1969).

Of these potential limitations on survival in hypersaline waters, of particular interest in Chapter 3 is the luminal accumulation of Mg^{2+} and SO_4^{2-} as Na^+, Cl^-, and water are absorbed by the intestinal epithelium. Because of the relative impermeability of the epithelium to divalent ions (Hickman, 1968; Shehadeh and Gordon, 1969), these divalent ions become the dominant electrolytes in the luminal fluids in high salinity, potentially limiting water absorption by the intestinal epithelium due to the proportion of permeable and impermeable ions available within the luminal substrate. While Ca^{2+} is similarly impermeable and remains within the lumen, the formation of $CaCO_3$ precipitates in the teleost gut is well known and removal of this cation by precipitation thus contributes to a partial decrease of the luminal osmolality (Wilson et al., 2002), whereas concentration of Mg^{2+} and SO_4^{2-} increase osmolality. Chapter 3 tests the hypothesis that fish will have greater hypersalinity tolerance if the limitations on intestinal fluid transport are reduced by altering luminal concentration of $MgSO_4$. While the impact of $MgSO_4$ independent of NaCl has been investigated with respect to kidney function and urine production (McDonald and Walsh, 2007), this is the first study to consider the impact of $MgSO_4$ concentration within the intestinal lumen on water balance.

Most fish species inhabit apparently stable environments with respect to salinity. Thus, it is common to think of fish as either marine or freshwater inhabitants, but in fact, fluctuations in most environments expose fish to a range of salinities. The toadfish, for example, is a relatively stenohaline species, and would therefore be predicted to have minimal tolerance of salinity fluctuations. However, this species is naturally exposed to a wide salinity range in its home territory of Biscayne Bay (Lorenz and Serafy, 2006), with

a maximum mean bay-wide salinity reaching 41.8 ppt (Kelble et al., 2007), and toadfish have been observed in smaller areas with means of up to 59.3 ppt salinity (Ley et al., 1999). Considering these characteristics of its natural environment, it is perhaps not surprising that, although it inhabits only the marine environment, *O. beta* is known to maintain normal water and salt balance between 5 and 50 ppt seawater, and can survive for at least 7 days in 70 ppt seawater (McDonald and Grosell, 2006). The metabolic costs of osmoregulation are significant, and animals exposed to conditions outside of their normal operating levels, such as hypersalinity, may be adversely impacted by the stress and additional energy required by osmoregulatory processes. In general, fish species span a range of potential salinity tolerance, which often does not correlate to the natural salinity exposure undergone by each species. Thus, the ability of fishes to transition between the two distinct regulatory systems characteristic of teleosts in either seawater or freshwater is an essential trait, and study of this transition allows for better understanding of each system and its most essential components for water and ion transport.

While environmental salinity fluctuations are often over a relatively small range, some areas undergo dramatic salinity changes in short time scales. Fish that inhabit areas that experience high salinity are exceptionally well-adapted to tolerate salinity fluctuations. For example, the killifish, *Fundulus heteroclitus*, which lives in nearshore salt marshes that experience daily tidal fluxes, is capable of maintaining homeostasis in salinities ranging from freshwater up to 114 ppt seawater (Griffith, 1974) and can adjust to shifts in this range in a matter of hours. In addition to fish species that are exposed to hypersaline environments due to changes in their environment, a small group of species

also are required to adjust to salinity variations of their own making due to migrations that are part of their life history. This group includes the anadromous rainbow trout, *Oncorhynchus mykiss*.

Chapter 4 examines the effect of transfer to increased salinity on *O. mykiss*. Although the stress of salinity transfer can be fatal for trout following transfer to full-strength seawater (Landless, 1976; Johnston and Cheverie, 1985), longer acclimation time and/or larger fish size decreases this stress (Johnston and Cheverie, 1985; Fuentes et al., 1997) and rainbow trout of all sizes are able to tolerate transfer to salinities below seawater (up to 28 ppt salinity) (Landless, 1976; Eddy and Bath, 1979). Trout transferred to seawater experience an initial "crisis" period (8-30 h) during which the majority of ion influx and other physiological changes occur in response to the onset of drinking, followed by a longer stabilization period where more permanent regulatory changes take place, such as increased activity of intestinal and branchial Na^+-K^+-ATPase (Colin et al., 1985; Johnston and Cheverie, 1985; Grosell et al., 2007), and at 7-10 days post-transfer are considered fully acclimated (Bath and Eddy, 1979). The steelhead strain of trout smolts used for the study described in Chapter 4 are capable of being acutely transferred to 2/3 seawater and maintained indefinitely at this salinity with little mortality.

Chapter 4 considers the physiological and transcriptional changes which occur following transfer from freshwater to 70% seawater. Work on rainbow trout at Bamfield Marine Centre, BC, Canada included measurements of intestinal ion transport using isolated tissue. While the physiological data indicates the functional adjustments occurring in the intestinal epithelia in response to salinity transfer, the regulation of ion transporters as part of and in addition to the currently accepted transport model (Grosell

et al., 2009a) were investigated in all osmoregulatory organs (gill, kidney, gastrointestinal tract). In particular, three transporters (SLC26a6, Na^+-H^+ exchanger 1 (NHE1), and Na^+-H^+ exchanger 2 (NHE2)) were hypothesized to be important to intestinal ion transport. Thus, quantitative real-time PCR was used to consider the potential post-transfer transcriptional regulation of ion transporters important to both intestinal water uptake and maintenance of acid-base balance.

In contrast to the anadromous trout or stenohaline toadfish, the euryhaline killifish (*Fundulus heteroclitus*) can easily acclimate to a broad range of salinities. In Chapter 5, the mechanism supporting this large salinity tolerance range is considered by comparisons of intestinal transport in killifish acclimated to either 35 or 70 ppt seawater. *In vivo* samples of intestinal fluids from fish acclimated to hypersalinity were examined for ion transport characteristics, including intestinal base secretion, which enhance water absorption in osmoregulatory challenged fish. Observation of these transport characteristics in isolated tissue was difficult, as the tissue is not viable *in vitro* under asymmetrical conditions. However, *in vitro* preparations were able to clarify some of the primary transport mechanisms important for intestinal osmoregulation in the killifish, particularly with regard to salinity increases. Killifish may have a large tolerance for salinity change because the mechanisms important for maintenance of water, ion, and acid-base balance under hypersaline and fluctuating conditions are permanent fixtures of the intestinal tissue in this tidal zone inhabitant.

The research comprising this dissertation investigates the dynamic regulation and physiological mechanisms of intestinal osmoregulation, particularly intestinal base secretion, in teleost fish exposed to increased ambient salinity. In four research chapters I

compare the effects of salinity change on three fish species with differing environmental exposure and tolerance to salinity: the gulf toadfish (*Opsanus beta*), rainbow trout (*Oncorhynchus mykiss*), and killifish (*Fundulus heteroclitus*). Comparison of the osmoregulation-linked ion and fluid transport in the relatively stenohaline gulf toadfish, the anadromous rainbow trout, and the highly euryhaline killifish allows investigation of the mechanisms that dictate tolerance to increased salinity and its role in ion, water, and acid-base balance in teleost species.

Chapter 2

Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*

Summary

Marine teleosts have extracellular fluids less concentrated than their environment resulting in continual water loss and compensate by drinking, with intestinal water absorption driven by NaCl uptake. Absorption of Cl^- occurs in part by apical Cl^-/HCO_3^- exchange, with HCO_3^- provided by transepithelial transport and/or by carbonic anhydrase-mediated hydration of endogenous epithelial CO_2. Hydration of CO_2 also liberates H^+, which is transported across the basolateral membrane. In this study, gulf toadfish (*Opsanus beta*) were acclimated to 9, 35, and 50 ppt salinities. Intestinal HCO_3^- secretion, water and salt absorption, and the ensuing effects on acid-base balance were examined. Rectal fluid excretion greatly increased with increasing salinity from 0.17±0.05 ml kg^{-1} h^{-1} in 9 ppt seawater to 0.70±0.19 ml kg^{-1} h^{-1} in 35 ppt seawater and 1.46±0.22 ml kg^{-1} h^{-1} in 50 ppt seawater. Rectal fluid composition and excretion rates allowed for estimation of drinking rates, which increased with salinity from 1.38±0.30 to 2.60±0.92 and 3.82±0.58 ml kg^{-1} h^{-1} in 9, 35, and 50 ppt salinities, respectively. In contrast, the fraction of imbibed water absorbed decreased from 85.9±3.8% in 9 ppt seawater, to 68.8±3.2% in 35 ppt seawater, and 61.4±1.0% in 50 ppt seawater. Despite large changes in rectal base excretion from 9.3±2.7 to 68.2±20.4, and 193.2±64.9 μmol kg^{-1} h^{-1} in 9, 35, and 50 ppt seawater, respectively, acute or prolonged exposure to altered salinities was associated with only modest acid-base balance disturbances. Extra-intestinal, presumably branchial, net acid excretion increased with salinity (62.0±21.0, 229.7±38.5, 403.1±32.9 μmol kg^{-1} h^{-1} at 9, 35, and 50 ppt seawater, respectively),

demonstrating a compensatory response to altered intestinal base secretion associated with osmoregulatory demand.

Background

Marine teleosts maintain the osmotic pressure of their extracellular fluids at ~300-350 mOsm, approximately 1/3 that of seawater (~1000 mOsm) (Shehadeh and Gordon, 1969). This results in a constant diffusive salt gain from the surrounding environment, as well as osmotic water loss, for which fish compensate by drinking seawater. Water absorption is driven by active uptake of ions by the gastrointestinal tract (Smith, 1930), particularly Na^+ and Cl^- absorption across the intestinal epithelium (Skadhauge, 1974; Ando et al., 1975). A large portion of intestinal Cl^- absorption results from apical Cl^-/HCO_3^- exchange (Ando and Subramanyam, 1990; Wilson et al., 1996; Grosell et al., 2001; Grosell et al., 2005), which occurs in excess and independently of Na^+ absorption (reviewed by Grosell, 2006). The secreted HCO_3^- has two sources: hydration of endogenous metabolic CO_2 (Wilson and Grosell, 2003; Grosell et al., 2005; Grosell and Genz, 2006) mediated by carbonic anhydrase (Grosell et al., 2005), and extracellular HCO_3^- transport across the basolateral membrane, presumably by Na^+/HCO_3^- cotransport (Ando and Subramanyam, 1990; Grosell and Genz, 2006). The contribution of HCO_3^- derived from each of these two sources varies among species (Grosell, 2006), but each accounts for approximately 50% of intestinal HCO_3^- secretion in the gulf toadfish, *Opsanus beta* (Grosell and Genz, 2006). In *O. beta*, the H^+ liberated by intracellular carbonic anhydrase-mediated CO_2 hydration is transported across the basolateral membrane into the extracellular fluids (Grosell et al., 2001) by an NHE-like basolateral

transport process (Grosell and Genz, 2006) and results in net acid absorption (Grosell and Taylor, 2007) proportional to the HCO_3^- secretion driving intestinal fluid absorption.

Fish maintain relatively alkaline blood pH ranging in most cases from 7.7 to 8.1 depending on temperature, primarily by transfer of acid-base molecules across the gill epithelium by mitochondrion-rich cells (Marshall and Grosell, 2005; Evans et al., 2005). Due to high solubility in water, the unidirectional movement of water across the respiratory surface and the countercurrent blood and water flow at the gill, molecular CO_2 from the blood is readily excreted into the water during gas exchange with carbonic anhydrase-rich erythrocytes, resulting in low plasma P_{CO2} compared to air-breathing vertebrates (Heisler, 1980; Claiborne, 1997). Hyperventilation to combat acidosis is therefore a relatively inefficient strategy in water-breathing compared to airbreathing animals due to the lower scope for change in P_{CO2}. Instead, the main response to a metabolic acidosis in teleost fish is increased acid excretion across the gill epithelium (McDonald et al., 1982; Evans, 1982; Claiborne, 1997). Both Na^+/H^+ exchange and V-type H^+-ATPase excrete H^+ from the gill, while the primary mechanism for base excretion at the gill is apical Cl^-/HCO_3^- exchange (Claiborne et al., 1997; Claiborne et al., 2002).

Changes in intestinal base secretion rates associated with osmoregulatory processes would be expected to have an impact on whole-animal acid-base balance. Higher drinking rate in elevated salinity is predicted, due to greater diffusive fluid loss, and fish exposed to high salinity are also predicted to increase HCO_3^- secretion into the intestinal lumen to facilitate water absorption (McDonald and Grosell, 2006; Grosell, 2006). Increased intestinal HCO_3^- secretion creates a potential problem, as it results in

systemic acid gain and the possibility of metabolic acidosis. A compensatory mechanism may exist to avoid disturbance of systemic acid-base balance caused by changes in intestinal base secretion, and, if present, this mechanism would likely occur at the gill, the primary acid-base regulatory tissue in fish.

To investigate the impact of high or low salinity on HCO_3^- secretion into the intestinal lumen, and subsequent systemic acid-base consequences, we collected the rectal fluids excreted by gulf toadfish acclimated to 9, 35, and 50 ppt salinities. The extracellular fluids of the fish are isoosmotic to the surroundings at 9 ppt seawater, reducing the need to drink. Conditions naturally experienced by the gulf toadfish are represented by 35 ppt seawater, while 50 ppt seawater represents a high salinity tolerance limit, intended to osmotically stress the fish and increase intestinal HCO_3^- secretion above usual rates (McDonald and Grosell, 2006) without causing severe disturbance of salt and water balance. As predicted, ambient salinity was observed to strongly influence rectal base excretion and the hypothesis of extra-intestinal compensation was therefore examined by measurements of net, extra-intestinal acid fluxes at 9, 35, and 50 ppt salinities.

Materials and Methods

Experimental Animals

Gulf toadfish (*Opsanus beta*) were obtained as by-catch from Biscayne Bay, FL, US by shrimp fishermen and transferred to 62 l aquaria at the Rosenstiel School of Marine and Atmospheric Science. Immediately after transport, fish received a prophylactic treatment to remove ectoparasites (McDonald et al., 2003). Toadfish were held in tanks which had

continuous flow of aerated, filtered seawater from Biscayne Bay (Bear Cut, 34-37 ppt seawater, 22-26˚C) for at least two weeks before experimentation. Segments of polyvinyl chloride tubing were provided for shelter and the fish were fed pieces of squid until satiation twice weekly, but starved for 48 h prior to sampling. Fish were maintained in the lab and used according to an approved University of Miami animal care protocol (IACUC #05-251).

Determination of drinking rate

The method used to determine drinking rate in toadfish acclimated to 50 ppt seawater (n=8) was adapted from Grosell *et al.* (2004). Fish were allowed to acclimate overnight to a smaller (5 l) glass container to facilitate efficient isotope use, after which water flow was stopped and 10 μCi [^{14}C] polyethylene glycol-4000 (PEG-4000) l^{-1} (specific activity: 2050 μCi/g; NEN-Dupont, Boston, MA, US) was added to the tank. Following a 6.1 h exposure, during which water samples were taken at 0.25 and 6.1 h, tricaine methane sulfonate (MS-222) was added to the tank for a final concentration of 0.2 $g \cdot l^{-1}$, euthanizing the fish which were removed individually from the tank, rinsed with non-radioactive 50 ppt seawater, weighed (22.3±1.3 g), and sampled. Sampling consisted of clamping the gastrointestinal tract at the start of the esophagus and immediately anterior to the anus by hemostats to prevent loss of fluid, and removal of the entire gastrointestinal tract from the body. Gastrointestinal samples were weighed, homogenized and digested in 10 ml H_3PO_4 (3% v/v) and prepared for analysis by combining 1 ml aliquots of supernatant from each homogenized sample with 4 ml of 50 ppt seawater. A ^{14}C-free 5 ml 50 ppt seawater sample served as blanks for these

analyses. A 1 ml aliquot of each water sample was diluted to 5 ml total volume with 50 ppt seawater; β radioactivity was determined for all samples by liquid scintillation counting using a TmAnalytic BetaTract 6895 instrument (Elk Grove Village, IL, US). No quenching was observed. Drinking rate (ml kg^{-1} h^{-1}) was calculated from the radioactivity in digested gastrointestinal tissue samples and that of water samples, weight of individual fish including the weight of the gastrointestinal tissue, and exposure time. The total activity of the GI tract was determined from the total volume of tissue homogenate (mass of tissue and fluid + H_3PO_4) and the activity recorded in the 1 ml aliquot. Note that this procedure has been verified with respect to absence of ^{14}C-PEG in plasma and rectal fluids at the end of the 6 hour incubation period for toadfish exhibiting drinking rates as high as in the present study (Grosell et al., 2004).

Acute transfer to hypersalinity

Toadfish were kept in a 103 l aquarium with filtered seawater (~35 ppt seawater) on a flow-through system, as described above. Water flow was terminated and approximately 80% of the water was siphoned off and replaced with a mixture of sea salt (Instant Ocean, Aquarium Systems Inc., Mentor, OH, US) dissolved in 35 ppt seawater to give a final salinity of 60 ppt. Fish were acutely transferred to 60 ppt seawater, as it was observed that this procedure could be tolerated by non-cannulated fish. Fish (n=8) were sampled immediately before transfer to 60 ppt seawater and at 6, 24, and 96 h post-transfer. Blood samples (~200 µl) were obtained by caudal puncture with a heparinized 1 ml syringe (BD Syringe, Franklin Lakes, NJ, US) fitted with a 21-gauge needle and placed on ice; plasma samples were promptly obtained by centrifugation (3 min at 10000 g)

(Eppendorf 5415D, Hamburg, Germany). Following anesthesia in 0.2 $g \cdot l^{-1}$ MS-222, fish were immobilized and euthanized by cutting the spinal cord and piercing the brain and the gastrointestinal tract was subsequently exposed by dissection. Note that this procedure rarely results in anal emptying when sampling occurs immediately after euthanasia. The intestine was clamped immediately anterior to the rectum and removed from the body cavity, after which the intestinal contents were emptied into sample tubes for analysis, detailed below.

Gradual acclimation to low and high salinities

Groups of 6-8 toadfish were acclimated over a period of two weeks to 9, 35, and 50 ppt salinities under static renewal conditions in tanks fitted with biological filters and aeration. Natural seawater from Bear Cut is typically 35 ppt, while 9 and 50 ppt salinities represent the lower and higher salinity tolerances of cannulated toadfish. Water was changed every two days by siphoning off water (~80%) and detritus and replacing this volume with water of appropriate salinity, either diluted or concentrated by addition of reverse osmosis water or Instant Ocean sea salt, respectively, to acclimate fish to low and high salinities. Salinities of the exposure waters were monitored by the use of a refractometer and resulting Na^+, Cl^-, Mg^{2+} and SO_4^{2-} were (all in mM) 112, 133, 12, 5.3, respectively for the 9 ppt treatment, 454, 440, 46, 24, respectively for the 35 ppt treatment and finally 797, 649, 87 and 32 mM, respectively for the 50 ppt treatment. Fish were fed to satiation every two days; continued appetite was considered an indication of acclimation with minimal stress. Measurements of ammonia concentrations in water from the holding tanks revealed total NH_4^+ concentrations of less than 112 μM in all cases.

Cannulation of acclimated toadfish

Following acclimation to 9, 35, and 50 ppt seawater, toadfish were exposed to 0.2 $g \cdot l^{-1}$ MS-222 in the same salinity water to which they were acclimated until anaesthetized and gills were perfused with 0.1 $g \cdot l^{-1}$ MS-222 in the appropriate salinity throughout surgery. A caudal incision allowed for insertion of a catheter of polyethylene tubing (PE 50) (Intramedic, Becton Dickinson & Co., Sparks, MD, US) into the caudal artery or vein. The catheter was enclosed in a short sleeve of larger tubing (PE 160), the exposed segment of which was secured to the skin by silk ligature, anchoring the catheter in the muscular tissue. The caudal incision was treated with antibiotic (oxytetracyclin) before being closed with silk ligatures. The catheter was filled with heparinized Hanks saline (50 i.u. ml^{-1}) (Walsh, 1987; Wilson and Grosell, 2003) and sealed. Each fish was also fitted with a rectal collection sac consisting of a latex balloon securely tied to a 1 cm segment of a 1 ml syringe (BD Syringe), heat-flared at both ends. The open end of the syringe segment was inserted into the anus and held in place by a purse-string ligature, allowing rectal contents to drain continuously into the balloon.

Immediately after surgery, fish were placed in individual flux chambers containing a known volume (~1 l) of seawater at their acclimation salinity. Following a recovery period, during which fish resumed their usual activity level and behavior (~10 min), initial water samples were taken for analysis of ammonium and total titratable base. Fish were kept in these aerated flux chambers for 24 h, at which point final water samples were collected for 24 h flux measurements. The flux chambers were then flushed with clean water of the appropriate salinity and a second 24 h flux was initiated. At the end of this second 24 h flux, water samples were taken, blood was sampled (~200 µl) via the

caudal catheter, and fish were euthanized with an overdose of MS-222. The contents of the rectal sacs were collected into pre-weighed 50 ml Falcon tubes, fish were weighed, and intestinal contents (fluid and precipitate) were collected as described above for the acutely transferred fish, in pre-weighed 15 ml Falcon tubes.

Analytical Techniques

Intestinal and rectal samples were centrifuged to obtain solid matter and the fluid was transferred into separate pre-weighed sample tubes by pipetting. The total amount of intestinal and rectal content and the proportions represented by fluid and precipitate were determined by weight. Total bicarbonate/carbonate content of intestinal precipitate was determined by double-endpoint titration. Samples of precipitate isolated from rectal and intestinal fluids were prepared for titration by homogenization and resuspension in 5 ml deionized water. The prepared sample was continuously aerated with N_2 gas, titrated to pH 3.80 with 0.02 N HCl and then titrated back to the initial pH using 0.02 N NaOH. For some precipitate samples, 0.2 N HCl and 0.02 N NaOH were used in order to minimize the volume of acid addition. The pH of the sample was monitored using Ag/AgCl combination electrodes (Radiometer Analytical, PHC 3005-8, Lyon, France) and a pH meter (Radiometer, PHM201). Acid and base were added using 2.0 ml micrometer syringes (Gilmont Instruments, GS-1200, Barrington, IL, US). In addition to analyzing ionic composition of the intestinal and rectal fluids phase, the solutions resulting from the titrations of rectal pellets were also analyzed for Mg^{2+} and Ca^{2+} to determine the content of these ions in the rectal pellets. The fraction of Mg^{2+} and Ca^{2+} eliminated from the

rectum in precipitates was calculated by relating the amount present in the pellets to the total amount of these ions eliminated via fluids and pellets combined.

Double endpoint titrations were also done on initial and final water samples (5 ml) from the flux chambers to determine total titratable acid flux for each 24 h period. Blood plasma and intestinal fluids of cannulated toadfish were analyzed for pH in contact with atmospheric air (Accumet 13-620-96 microelectrode Fisher Scientific, Pittsburg, PA, US, coupled to a Radiometer PHM201 pH meter), and for total CO_2 using a total CO_2 analyzer (Corning 965, Medfield, MA, US). Blood plasma collected from acutely transferred fish was not measured for pH, but only total CO_2, given the limitations of the caudal puncture technique. Anion concentrations from all fluid samples were quantified by anion chromatography (Dionex 120, Sunnyvale, CA, US), while cations were analyzed by fast sequential flame atomic absorption spectrometry (Varian 220, Palo Alto, CA, US) using an air/acetylene flame. Ammonium content of water samples was determined by colorimetric assay from Verdouw *et al.* (1978), modified for microplates using standards made up in solutions of the appropriate salinity.

Bicarbonate equivalents in blood plasma, intestinal fluids and rectal fluids for acclimated, cannulated fish were determined from the total CO_2 and pH measurements using the Henderson-Hasselbalch equation. More specifically, the HCO_3^- concentration was determined from equation I while the CO_3^{2-} concentration was determined from equation II, in both cases using a pK_{II} of 9.46 for toadfish gut fluids (Wilson et al., 2002).

Equation I:

$[HCO_3^-] = [\text{total } CO_2]/(1+10^{pH-pK_{II}})$

Equation II:

$[CO_3^{2-}] = [\text{total } CO_2] - ([\text{total } CO_2]/(1+10^{pH-pK_{II}}))$

Note that this approach ignores the contribution from molecular CO_2 which is justified by molecular CO_2 concentrations of less than 10^{-9} M under conditions relevant for the present study (Grosell et al., 2005). The HCO_3^- equivalents of fluid samples measured in the present study were calculated as the sum of $[HCO_3^-]$ and $2x[CO_3^{2-}]$. Note that this approach has been established previously showing strong correlation between results obtained from the above calculations and double endpoint titrations (r^2 >0.999) (Grosell et al., 1999).

Extra intestinal fluxes of acid-base equivalents were determined from the change in water concentrations of titratable acid and total ammonia during the two sequential 24 h periods, the volume of water in the flux chambers, the fish mass and the exact time elapsed. Rectal base output rates were determined from the total amount of base present in the excreted precipitates (determined by double endpoint titrations) and the total HCO_3^- equivalents in the excreted fluids (calculated from total CO_2 and pH) at the end of the 48 hour experimental period.

Data presentation and statistical analysis

All values are given as mean ± s.e.m. The control (0 h post-transfer in acutely transferred toadfish, or 35 ppt seawater in acclimated toadfish) and experimental values were compared using one-way ANOVA, followed by Bonferroni-corrected t-tests to compare individual means to the control value. Data sets found not to be normally distributed were compared using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method. Differences between means were considered significant when $P<0.05$.

Results

Drinking rate and rectal excretion

In this study, the drinking rate of *O. beta* in 50 ppt seawater was measured to be 3.24±0.34 ml kg^{-1} h^{-1}. The total fluid volume excreted from the rectum during the 48 h of experimentation was 0.17±0.05 ml kg^{-1} h^{-1} in 9 ppt seawater, 0.70±0.19 ml kg^{-1} h^{-1} in 35 ppt seawater, and 1.46±0.22 ml kg^{-1} h^{-1} in 50 ppt seawater.

Acute Transfer to Hypersalinity

Toadfish acutely transferred from 35 to 60 ppt seawater displayed a transient disturbance of acid-base balance. Total CO_2 of blood plasma was significantly reduced at 96 h following transfer to 60 ppt seawater, while intestinal fluids demonstrated no change in total CO_2 (Fig. 2.1). In the intestinal fluid, Cl^- concentration was elevated at both 24 and 96 h post-transfer (Fig. 2.2), which coincides with the decrease to initial values seen in total CO_2 in the intestinal fluid at these time points (Fig. 2.1). In contrast, Na^+, Ca^{2+}, K^+, and SO_4^{2-} concentrations in the intestinal fluids were relatively stable over time.

Figure 2.1. Total CO_2 in the blood plasma (open circles) and intestinal fluid (closed circles) of toadfish acutely transferred from 35 to 60 ppt seawater and sampled at 0, 6, 24, and 96 h post-transfer. Mean ± s.e.m. (n≥7). An asterisk (*) indicates significant difference from 0 h value (P<0.05).

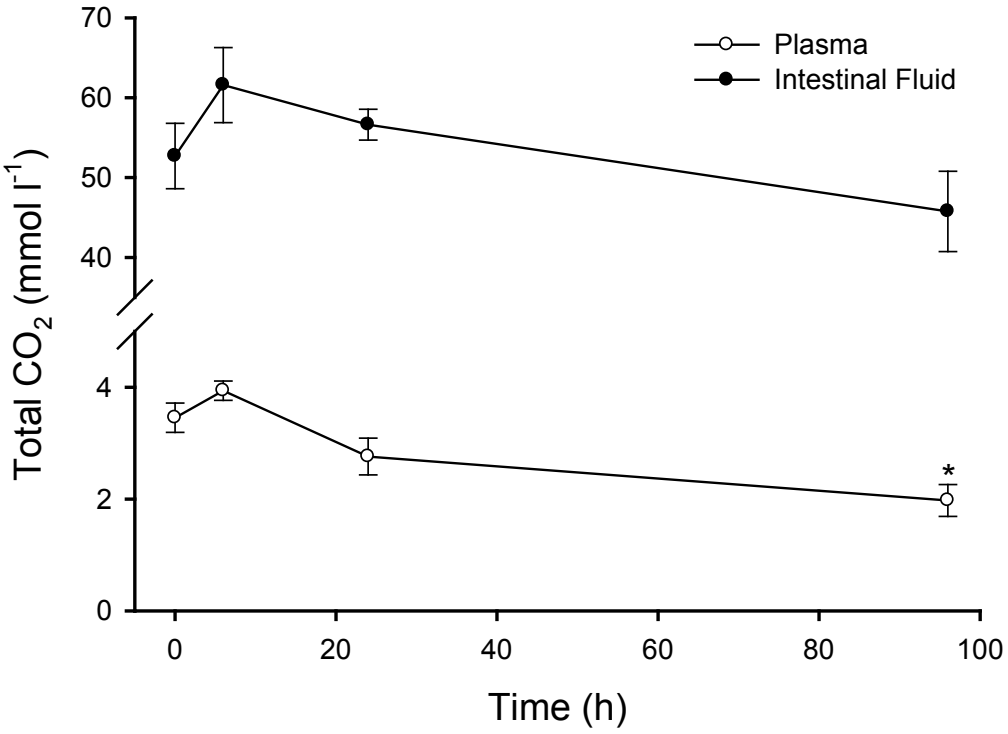


Figure 2.2. Concentrations (mM) of Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, Ca^{2+}, and K^+, in intestinal fluids of toadfish acutely transferred from 35 to 60 ppt seawater and sampled at 0, 6, 24, and 96 h post-transfer. Mean ± s.e.m. (n≥5). An asterisk (*) indicates significant difference from 0 h value (P<0.05).

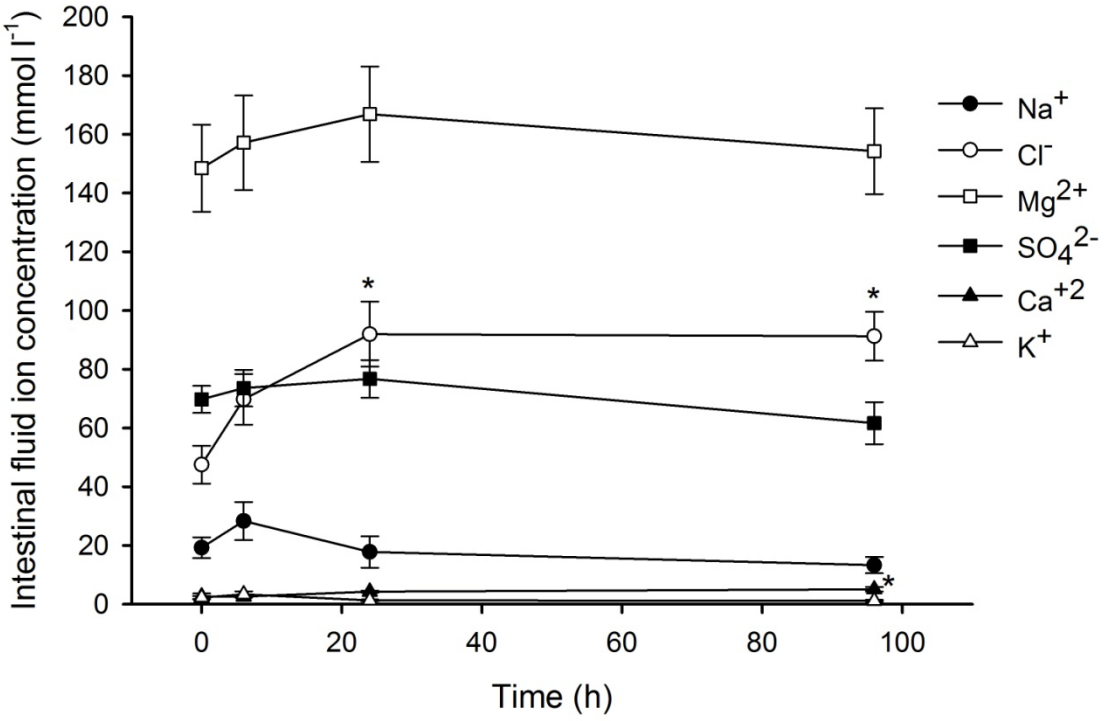
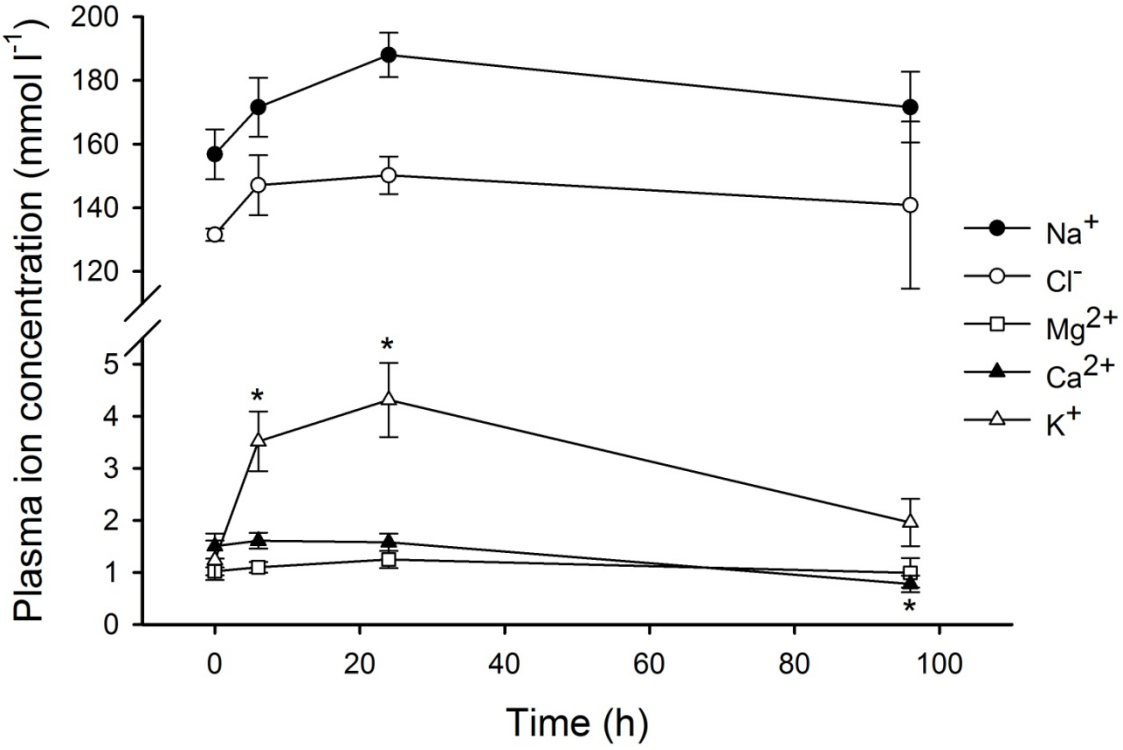


Figure 2.3. Concentrations (mM) of Na^+, Cl^-, Mg^{2+}, Ca^{2+}, and K^+ in blood plasma of toadfish acutely transferred from 35 to 60 ppt seawater and sampled at 0, 6, 24, and 96 h post-transfer. Mean ± s.e.m. (n≥6). An asterisk (*) indicates significant difference from 0 h value (P<0.05).



However, in the plasma of toadfish transferred acutely to 60 ppt seawater (Fig. 2.3), both Na^+ and Cl^- appeared to increase during the first 24 hours after transfer, while Ca^{2+} was significantly lower than control values after 96 h ($P<0.03$) and the concentration of K^+ increased greatly in the first 24 h, returning to initial levels by 96 h. The low plasma Mg^{2+} concentrations remained constant (Fig. 2.3) following transfer to 60 ppt seawater.

Gradual acclimation to low and high salinities

Plasma HCO_3^- equivalents in toadfish acclimated to both 9 ppt and 50 ppt seawater were not significantly different than in samples obtained from toadfish in 35 ppt seawater. Bicarbonate equivalents in the rectal fluid are relatively constant in all salinities (Fig. 2.4). Rectal fluid pH was alkaline in all samples but was significantly decreased in 50 ppt seawater and increased in isoosmotic conditions (9 ppt seawater) compared to control values (35 ppt seawater). Similarly, plasma pH was lower at 50 ppt seawater and higher at 9 ppt seawater, compared to 35 ppt seawater. Note that plasma samples reflect the status of the animals at the 48 h time point, whereas rectal fluid was accumulated over the entire 48 h period.

All measured ions in the plasma (Na^+, Cl^-, Mg^{2+}, Ca^{2+}, K^+) tended to be higher in 50 ppt than 35 ppt seawater (Table 2.1), although none of these differences were significant. In the rectal fluid of acclimated toadfish (Table 2.2), the concentrations of K^+ and Na^+ decreased from 9 to 35 ppt seawater, whereas SO_4^{2-} increased. Similarly, Mg^{2+} greatly increased both from 9 ppt to 35 ppt seawater, and from 35 ppt to 50 ppt seawater. The absolute ion excretion rate ($\mu mol\ kg^{-1}\ h^{-1}$) from the rectum over 48 h is shown in Fig. 2.5.

Figure 2.4. (A) HCO_3^- equivalents and (B) pH of blood plasma and rectal fluids collected at 48 h from toadfish acclimated to 9, 35, and 50 ppt salinities. Mean ± s.e.m. (n≥5). An asterisk (*) indicates significant difference from 35 ppt seawater (P<0.05).

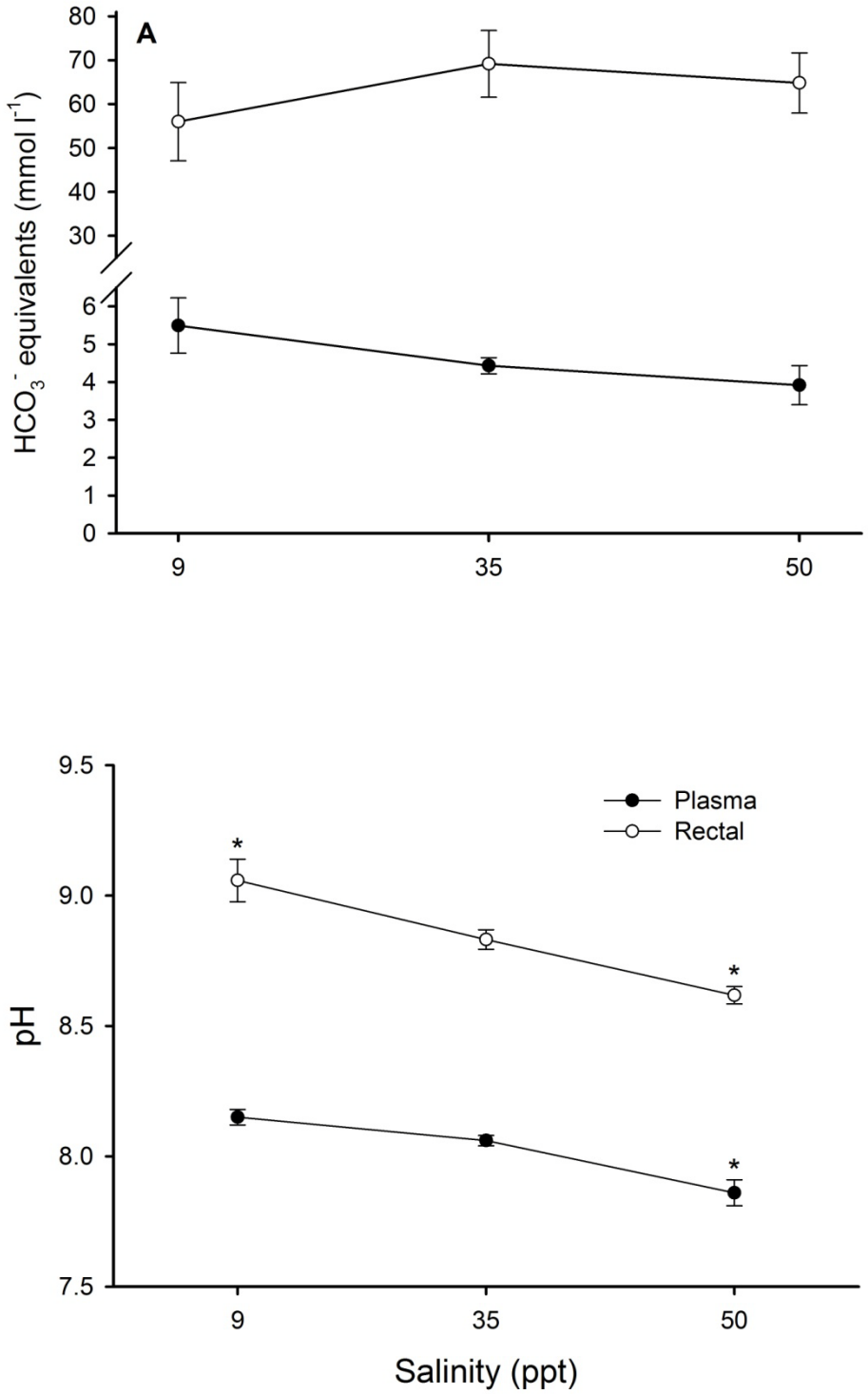


Table 2.1. Concentrations (mM) of Na^+, Cl^-, Mg^{2+}, Ca^{2+}, and K^+ in blood plasma of toadfish acclimated to 9, 35, and 50 ppt salinities for a minimum of 14 days. Mean ± s.e.m. (n≥4).

	Na^+	Cl^-	Mg^{2+}	Ca^{2+}	K^+
9	151.6 ± 5.1	132.1 ±19.0	0.6 ± 0.0	2.5 ± 0.2	2.8 ± 0.1
35	149.6 ± 2.0	136.6 ± 6.1	0.8 ± 0.1	2.3 ± 0.2	2.8 ± 0.2
50	165.7 ± 10.4	150.9 ±16.5	1.1 ± 0.3	2.6 ± 0.3	3.9 ± 0.9

Table 2.2. Concentrations (mM) of Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, Ca^{2+}, and K^+ accumulated over 48 h in rectal fluid of toadfish acclimated to 9, 35, and 50 ppt salinities. Mean ± s.e.m. (n≥4). An asterisk (*) indicates value is significantly different from 35 ppt seawater (P<0.05).

	Na^+	Cl^-	Mg^{2+}	SO_4^{2-}	Ca^{2+}	K^+
9	117.3 ± 17.5*	86.8 ± 10.2	46.3 ± 19.6*	48.8 ± 9.2*	2.0 ± 0.3	4.2 ± 0.6*
35	47.8 ± 19.4	90.1 ± 24.5	158.2 ± 16.8	83.2 ± 9.4	3.7 ± 0.5	1.6 ± 0.4
50	37.2 ± 15.2	132.2 ± 17.9	219.9 ± 6.3*	86.3 ± 2.7	3.5 ± 1.3	1.6 ± 0.6

Figure 2.5. Mg^{2+}, Cl^-, SO_4^{2-}, Na^+, Ca^{2+}, and K^+ excreted in rectal fluid over 48 h by toadfish acclimated to 9, 35, and 50 ppt salinities (μmol kg^{-1} h^{-1}). The fraction of total Mg^{2+} and Ca^{2+} excreted in precipitates are shown in stacked bars. Mean ± s.e.m. (n≥4). An asterisk (*) indicates significant difference from 35 ppt seawater (P<0.05).

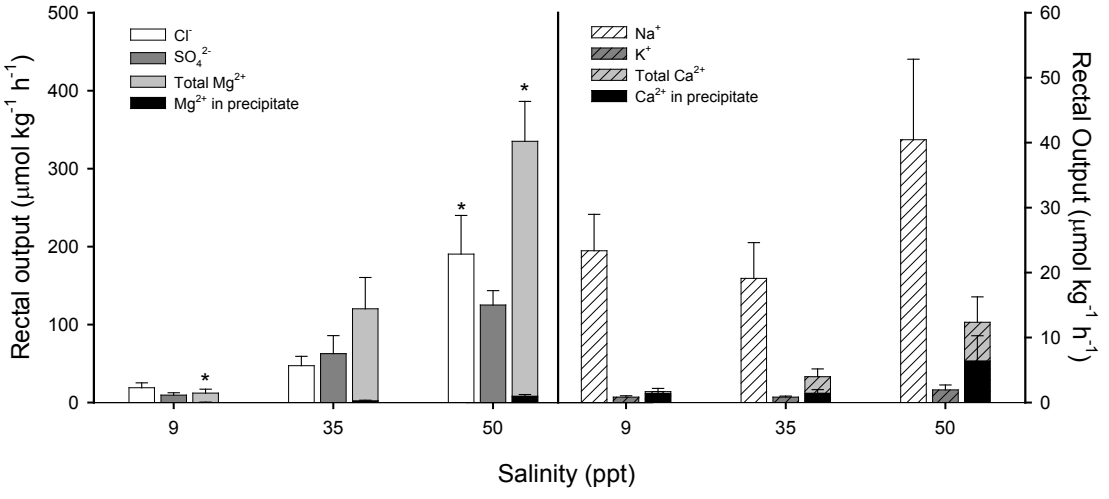
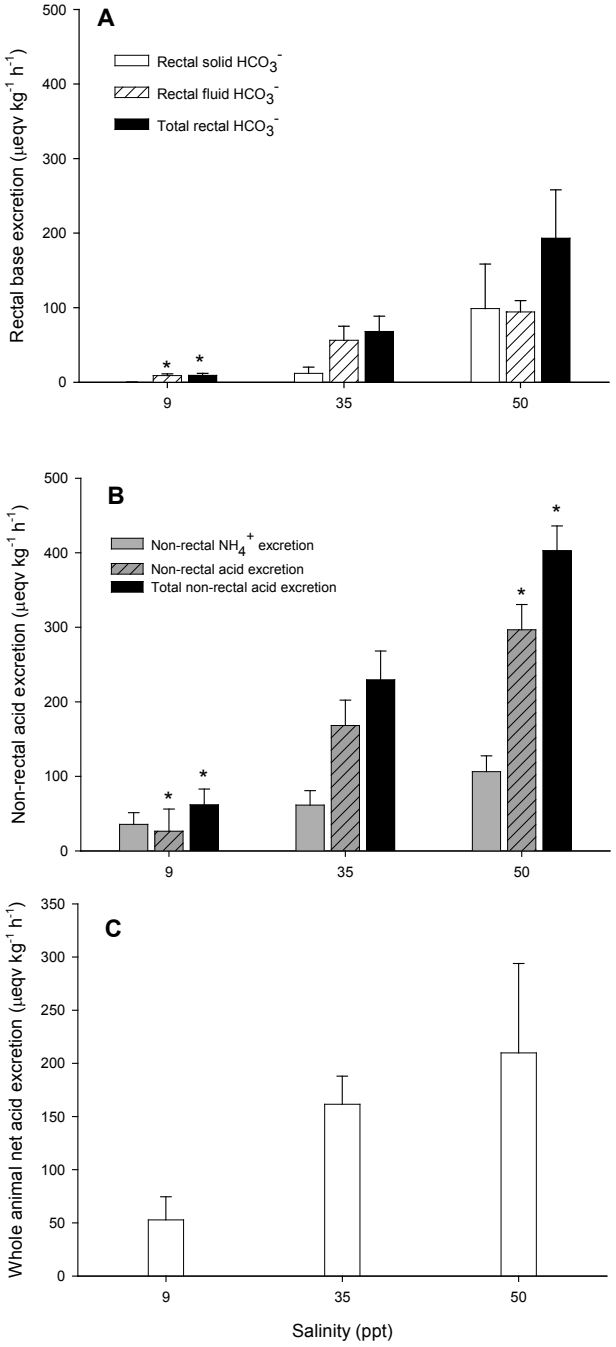


Figure 2.6. Rectal and non-rectal excretion of acid-base equivalents over 48 hours (μequiv kg^{-1} h^{-1}) by toadfish acclimated to 9, 35, and 50 ppt salinities and fitted with rectal collection sacs. (A) Total rectal base excretion is the sum of base equivalents in both rectal fluid and precipitate. (B) Total non-rectal acid excretion is the sum of non-rectal titratable acid and ammonium fluxes. (C) Whole-animal net acid-base flux is given as acid excretion, (the sum of the rectal base excretion and non-rectal acid excretion). Values are means ± s.e.m. (n≥6). An asterisk (*) indicates significant difference from 35 ppt seawater (P<0.05).



In toadfish acclimated to 50 ppt seawater, Mg^{2+} and Cl^- excretion was significantly greater than in 35 ppt seawater with a similar, nonsignificant trend for SO_4^{2-}, Na^+, K^+, and Ca^{2+}. Intestinal excretion of HCO_3^-, Ca^{2+}, and Mg^{2+} occurred both in solution and via precipitated solids. The amount of both Ca^{2+} and Mg^{2+} excreted as both fluid and precipitate increased with increasing salinity (Fig. 2.5). The fraction of Ca^{2+} excreted as precipitate increased from 26.8% in 35 ppt seawater to 61.2% in fish acclimated to 50 ppt seawater, while the percentage of excreted Mg^{2+} in the precipitate was very low (2.7% in 35 ppt seawater, and 3.6% in 50 ppt seawater). Rectal base excretion, as both HCO_3^- equivalents in rectal fluids and CO_3^{2-} precipitates, increased with salinity and the fraction of rectal base efflux occurring as precipitate increased from 16.7% in 35 ppt seawater to 23.4% in fish acclimated to 50 ppt seawater (Fig 2.6A). Non-rectal net acid excretion (sum of titratable acid flux and total ammonia flux) also increased with increasing salinity (Fig. 2.6B). Interestingly, ammonia excretion tended to increase with increasing salinity (P<0.062). Combined rectal base and non-rectal acid excretion rates indicate net acid excretion at the whole animal level (Fig. 2.6C), which displayed an increasing trend with increasing salinity.

Conclusions

Tolerance of elevated salinity

Increased water loss is certain to occur in fish exposed to hypersalinity and is compensated in part by increased HCO_3^- secretion by the intestine, resulting in marked effects on whole-animal physiology. The maximum and minimum salinity tolerance for *O. beta* has been determined previously; toadfish display normal plasma ion composition

between 5 and 50 ppt seawater, but not in 70 and 2.5 ppt seawater (McDonald and Grosell, 2006). Our results suggest that free-swimming, non-cannulated gulf toadfish can maintain normal regulation of plasma ionic composition in 60 ppt seawater for at least 96 h. However, a preliminary experiment in which fish were acclimated to 60 ppt seawater and underwent cannulation surgery resulted in high mortality. The acclimation salinity was therefore decreased to 50 ppt seawater for completion of cannulation experiments in the present study.

Increased drinking rate in elevated salinity

It is well-established that drinking rates of teleost fish increase with increased salinity (Shehadeh and Gordon, 1969; Sardella et al., 2004; Marshall and Grosell, 2005). Fish in hypo- or isoosmotic salinities have little need for water absorption across the gastrointestinal tract. Thus, as expected, the drinking rate of toadfish in isoosmotic conditions is low, demonstrated by the low rate of fluid excretion from the rectum at 9 ppt seawater compared to secretion rates from fish in 35 and 50 ppt seawater. Drinking rates were not determined directly in the present study in fish fitted with rectal catheters, but can be estimated from the volume of rectal fluid collected and the concentrations of Mg^{2+} and SO_4^{2-} in these fluids and in the ambient water. Such estimates rely on the assumption that the intestinal epithelium is relatively impermeable to $MgSO_4$ with little, if any, absorbed from the lumen by the intestine. This has been demonstrated previously (Hickman, 1968) and also is supported by recent findings of very low $MgSO_4$ absorption rates by isolated toadfish intestinal segments (Grosell and Taylor, 2007). Based on the concentration of Mg^{2+} in the rectal fluid, the volume of rectal fluid excreted, and

measured Mg^{2+} concentrations from all experimental salinities; we calculated drinking rates of 0.98±0.42, 2.56±0.87, and 3.75±0.59 ml kg^{-1} h^{-1} at 9, 35, and 50 ppt salinities, respectively. Corresponding estimates based on rectal concentrations of SO_4^{2-} and measured ambient SO_4^{2-} concentrations, are 1.79±0.55 (9 ppt seawater), 2.63±0.97 (35 ppt seawater), and 3.89±0.57 ml kg^{-1} h^{-1} (50 ppt seawater). Considering these observations of Mg^{2+} and SO_4^{2-} combined, drinking rates can be estimated to be 1.38±0.30 to 2.60±0.92 and 3.82±0.58 ml kg^{-1} h^{-1} in 9, 35, and 50 ppt salinities, respectively. No significant differences exist between the drinking rate estimates based on concentration of Mg^{2+} versus SO_4^{2-}, but drinking rates at 50 ppt seawater were significantly higher than at 9 ppt seawater according to both estimation methods. The mean estimated drinking rate based on rectal fluid excretion (of both Mg^{2+} and SO_4^{2-}) in 50 ppt seawater is not significantly different from that directly measured in non-cannulated toadfish acclimated to 50 ppt seawater (3.82±0.58 ml kg^{-1} h^{-1} compared to 3.24±0.34 ml kg^{-1} h^{-1}, respectively). The measured rate at 50 ppt seawater is higher than the mean rate estimated for cannulated fish in 35 ppt seawater (2.60±0.92 ml kg^{-1} h^{-1}) and higher than those previously reported for toadfish acclimated to 30 ppt seawater (Grosell et al., 2004). Admittedly, if $MgSO_4$ was absorbed by the intestine, our calculations would have underestimated the actual drinking rate. However, we note that our estimated drinking rates are well within the range established for seawater fish, reviewed by Marshall and Grosell (2005), and that measured and estimated drinking rates for the fish acclimated to 50 ppt seawater are similar.

It is known that cortisol, a key stress hormone, has a role in the regulation of drinking rate in teleost fish (Fuentes et al., 1996; Lin et al., 2000). Although transfer to

higher salinity increases drinking rate, a simultaneous addition of cortisol increases drinking rate to an even greater extent in both tilapia larvae (Lin et al., 2000) and juvenile rainbow trout (Fuentes et al., 1996). The estimated drinking rates for cannulated toadfish may thus be higher than those measured in non-cannulated fish due to the combination of high salinity and stress from handling and extensive surgical procedures.

Fractional fluid absorption

The difference between the estimated drinking rate (over ~48 h), and the measured rectal fluid excretion yields an estimate of total fluid absorption by the gut (Fig. 2.7). In fish acclimated to 35 ppt seawater, 68.8±3.2% (1.90±0.74 ml kg^{-1} h^{-1}) of the ingested seawater is absorbed by the gastrointestinal tract, which is within the range for seawater fish determined previously, which include 38.5% (Wilson et al., 2002), 75.8% (Hickman, 1968), 80% (Shehadeh and Gordon, 1969), and 84.9% (Wilson et al., 1996). Absorption values over the 48 h flux period were also estimated for toadfish in 9 ppt seawater (1.21±0.27 ml kg^{-1} h^{-1}) and 50 ppt seawater (2.36±0.36 ml kg^{-1} h^{-1}) to be 85.9% and 61.4% of the ingested volume, respectively. It is apparent that intestinal fluids in high salinity are depleted of NaCl, while $MgSO_4$ is concentrated to very high levels (Table 2.2). As mentioned previously, the intestinal epithelium has very low permeability to Mg^{2+} and SO_4^{2-} (Grosell and Taylor, 2007). Therefore, as salinity increases and permeable salts are absorbed, the dominant cation in the gastrointestinal fluids shifts from Na^+ to Mg^{2+} while the dominant anion shifts from Cl^- to SO_4^{2-} and HCO_3^-. As the ions available to drive water absorption are taken up by the epithelium and impermeable ions accumulate, it becomes increasingly difficult to absorb water from the concentrated fluid

in the lumen. This point is illustrated in Figure 2.8, which displays both the absorption rate of ingested Na^+ and Cl^-, as well as the fractional absorption of these ions. Absorption of these ions dramatically increases with salinity, although fractional absorption does not increase from 35 to 50 ppt seawater, apparently because fractional water absorption at 35 ppt seawater has already reached a maximum. The limitation of water absorption by extensive removal of Na^+ and Cl^- and increased concentration of impermeable, divalent ions in the intestinal fluids likely explains the observed decrease in the fraction of ingested water that is absorbed by the gastrointestinal tract with increasing salinity. It thus appears that since fractional water and NaCl absorption cannot increase at salinities higher than 35 ppt seawater, the only mode of response to the elevated diffusive fluid loss is increased drinking rate, as was observed in the present study.

Responses to acutely elevated salinity over time

Ionic composition of the intestinal fluid of toadfish acutely transferred from 35 ppt to 60 ppt seawater underwent a dynamic adjustment, which appeared to be stabilized by 24 h post-transfer. Intestinal fluid concentrations of Cl^- and Ca^{2+} at 24 and 96 h are significantly elevated from the initial (control) value which likely is an effect of increased drinking rate in response to increased salinity. The association between decreased HCO_3^- equivalents and increased Cl^- concentration in the intestinal fluids illustrates the importance of apical Cl^-/HCO_3^- exchange to intestinal osmoregulation. The stable concentration of Cl^- at 24 and 96 h most likely is the result of a constant seawater ingestion rate balanced by a constant Cl^- absorption rate, established during this period.

Figure 2.7. Estimated drinking rate, fluid absorption rate (ml kg^{-1} h^{-1}), and fractional fluid absorption of toadfish acclimated to 9, 35, and 50 ppt salinities. Drinking rates were estimated based on Mg^{2+} and SO_4^{2-} concentrations in rectal fluid samples and 9, 35, and 50 ppt seawater. Mean ± s.e.m. (n=6). An asterisk (*) indicates significant difference from 35 ppt seawater (P<0.05).

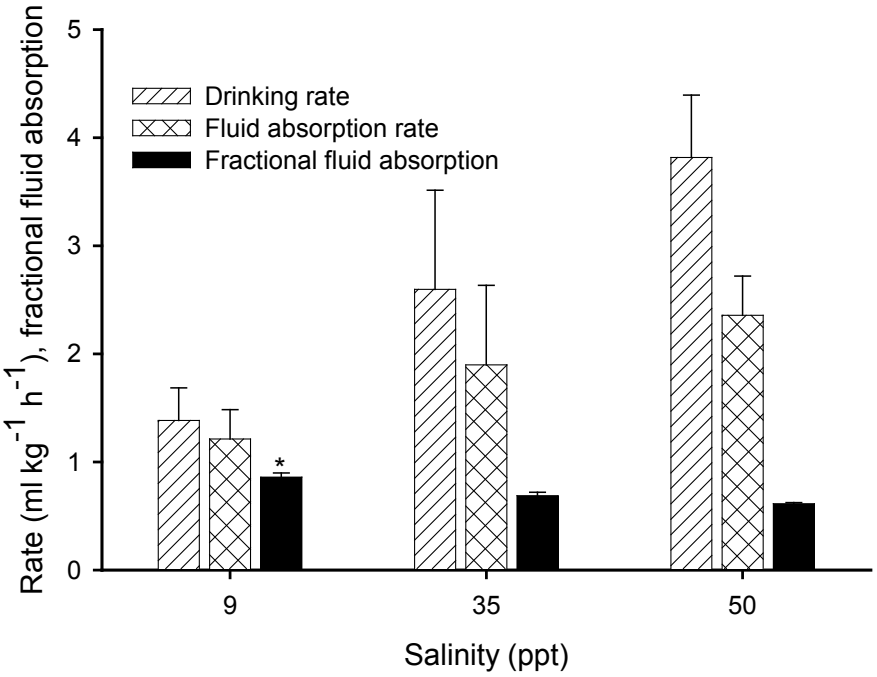
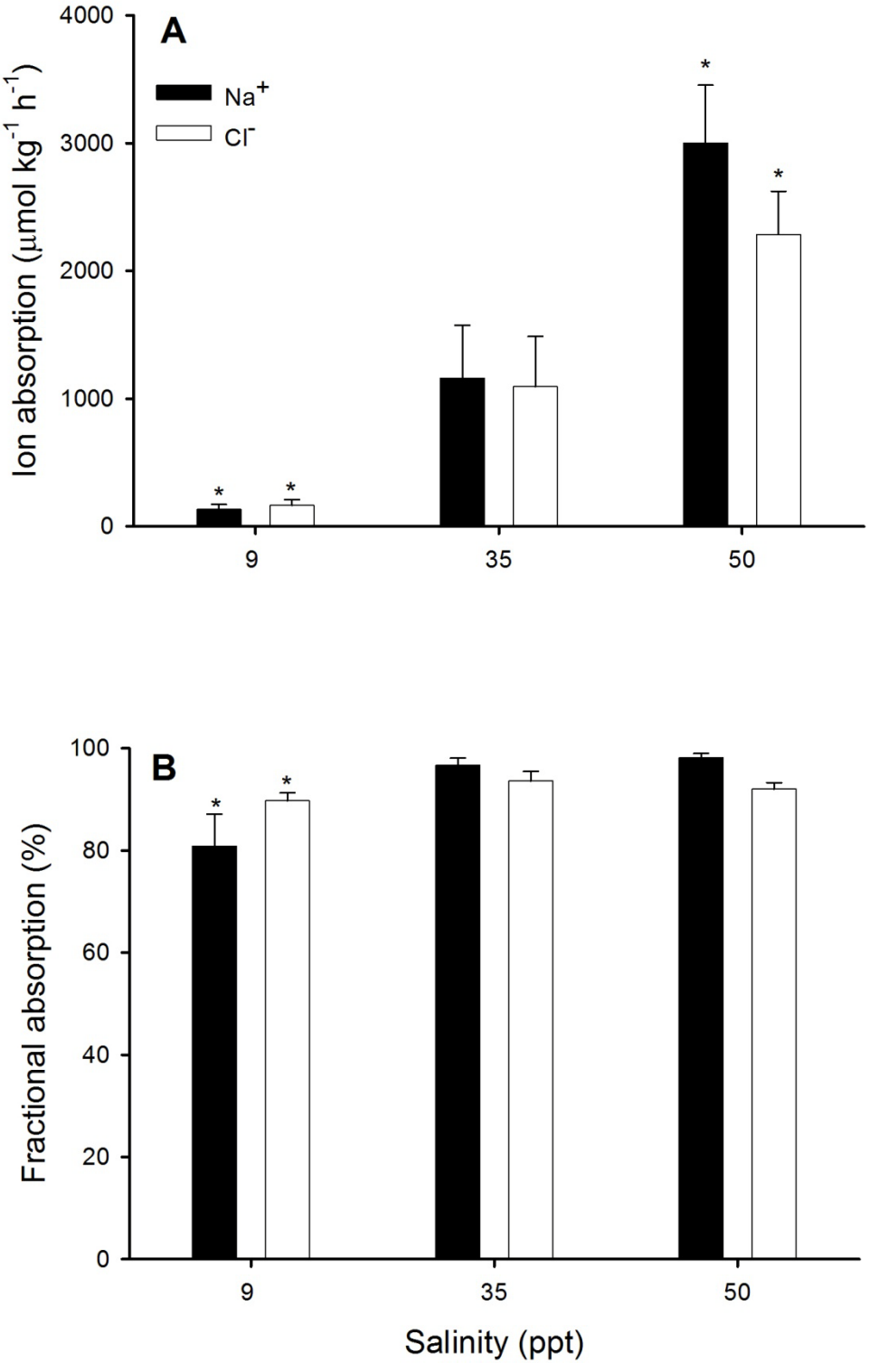


Figure 2.8. Calculated (A) absorption rate (μmol kg^{-1} h^{-1}) and (B) fractional absorption of ingested Na^+ and Cl^- by toadfish acclimated to 9, 35, and 50 ppt salinities based on Na^+ and Cl^- concentrations in rectal fluid samples and 9, 35, and 50 ppt seawater. Mean ± s.e.m. (n≥5). An asterisk (*) indicates significant difference from 35 ppt seawater (P<0.05).



Low Na^+ values, with a transient increase at 6 h, suggest an increased drinking rate, quickly followed by increased intestinal Na^+ absorption, the same process which seems to be impacting changes in Cl^- concentration. Apparently, the osmoregulatory response of Na^+ absorption occurs more quickly (by 24 h) than the time required for Cl^- uptake to adjust to high salinity conditions (between 24 and 96 h). This temporal separation is interesting, as Cl^- in part is absorbed by the intestine in exchange for an ion affecting acid-base balance (HCO_3^-), while Na^+ in the marine teleost intestine so far has not been demonstrated to be absorbed by transporters conducting movement of acid-base equivalents.

Acid-base response to elevated salinity

Rectal HCO_3^- equivalent excretion is higher in toadfish acclimated to 50 ppt than to 35 ppt seawater, indicating increased intestinal HCO_3^- secretion in response to increased drinking rate and osmoregulatory demand. Total base excretion increased with salinity, as did the proportion occurring as precipitate (3.3%, 16.7%, and 23.4% at 9, 35, and 50 ppt salinities, respectively). Consistent with this observation, plasma pH is significantly lower in toadfish acclimated to 50 ppt seawater than in fish acclimated to 35 ppt seawater, indicating an acid-base balance disturbance caused by increased HCO_3^- secretion into the intestinal lumen in response to elevated salinity. The mechanism for apical transport of HCO_3^- in the intestinal epithelium has been characterized and shown to occur in parallel with H^+ transport across the basolateral membrane in gulf toadfish (Grosell and Genz, 2006). Thus, when these transport processes occur to a greater extent in high salinity, an elevated net acid load in the extracellular fluids occurs. However, the

acid-base disturbance observed in toadfish acclimated to 50 ppt seawater is much less dramatic than could be expected given the intensity of the osmoregulatory challenge and the 21-fold increase in total rectal base efflux between 9 ppt seawater (9.3±2.7 μmol kg^{-1} h^{-1}) and 50 ppt seawater (193.2±64.9 μmol kg^{-1} h^{-1}). The limited nature of the whole-animal acid-base balance disturbance (modest changes in HCO_3^- equivalents and pH in extracellular fluids) is explained by adjustments occurring on the whole-animal scale which include markedly elevated extra-intestinal net acid excretion to maintain acid-base balance following salinity transfer (Fig. 2.6).

We note that our plasma pH values (8.06) are higher than values previously reported (~7.85) from toadfish in seawater (Barber and Walsh, 1993), a difference we ascribe to our plasma pH measurements not being performed under gastight conditions. To evaluate this potential influence of diffusive CO_2 loss from samples exposed to atmospheric air prior to pH measurements additional measurements were performed. Blood obtained via Hamilton syringes from toadfish (n=5) fitted with caudal catheters were analyzed immediately for pH in a custom made gas tight, water jacketed chamber fitted with a Radiometer combination pH electrode (4000-8). Subsequently, a fraction of these individual blood samples were subjected to the procedure used for the above analysis and plasma pH was measured under contact with atmospheric air. The pH values obtained using gastight measurements on whole blood were 7.824 ± 0.045, while the values obtained from air exposed plasma for a time period relevant to the original measurements were 7.992 ± 0.027. These follow up measurements thus suggest that air exposure of the plasma samples account at least in part for relatively high pH values obtained in the present study. A further complication of plasma pH measurements is the

mixture of arterial and venous blood collected via caudal puncture in fish acutely exposed to 60 ppt seawater. In fish acclimated to 9, 35, and 50 ppt waters, catheters resided in either the artery or vein, and impact of air exposure would be expected to be greater on venous than arterial samples. However, mean values were determined without attempting to distinguish the blood vessel source of the collected plasma. Regardless, samples from all experimental groups were treated the same such that differences among experimental groups should be robust.

Gill and kidney as possible sites of compensatory acid excretion

The gill is the primary organ responsible for regulation of acid-base balance in teleost fish. Another possible route for extra-intestinal acid excretion is the kidney. However, in the aglomerular toadfish, the kidney plays a modest regulatory role, even less so than the limited impact observed in glomerular fish (McDonald et al., 1982; Maren et al., 1992). Titratable acid fluxes determined as part of the present study demonstrate an increase in extra-intestinal, presumably branchial, net acid excretion in elevated salinity (Fig. 2.6). Thus, it seems that increased acid extrusion at the gill compensates for increased transport of H^+ into the extracellular fluids occurring in response to intestinal processes associated with high salinity. Further studies of the dynamics of this compensatory branchial acid excretion and the underlying mechanisms clearly are warranted.

Nitrogenous waste excretion at the gill

Just as the gill extruded more H^+ in elevated salinity, branchial NH_4^+ (or NH_3) excretion also appeared to be elevated. Under normal conditions, toadfish excrete nitrogenous

waste primarily as ammonia (McDonald et al., 2006). During periods of acute stress, however, nitrogenous waste excretion shifts to favor urea (Hopkins et al., 1995). The increased NH_3/NH_4^+ excretion observed in this study is contrary to what would be expected in stressed and confined toadfish and is therefore unlikely to be related directly to osmoregulatory and surgery-related stress, and may instead represent a response to the acid-base balance disturbance or the excess energy demand associated with an increased osmotic challenge. It has previously been observed that urea production decreases during hypercapnia (Barber and Walsh, 1993; McDonald et al., 2007). Increased intestinal HCO_3^- secretion in high salinity diminishes HCO_3^- concentration in extracellular fluids and it is clear that an osmoregulation-related acidosis, although modest, occurs in high salinity. It is plausible that the observed increase in branchial NH_3/NH_4^+ secretion may be a compensatory mechanism for regulation of acid-base balance. An increase in NH_4^+ rather than NH_3 excretion would be advantageous for maintenance of acid-base balance by serving the dual function of both acid excretion (as NH_4^+) and the retention of HCO_3^- which would otherwise be consumed during urea production (Atkinson, 1992). Excretion of NH_4^+ rather than incorporation of nitrogen into urea would allow both adequate excretion of nitrogenous waste and contribute to the correction of acid-base balance. Furthermore, it might act as a mechanism for energy conservation, as urea synthesis is an ATP-consuming process. It is generally assumed that nitrogenous waste excretion by ammoniotelic marine teleosts occurs via a paracellular route and that it is driven mainly by the blood side positive transepithelial potential (TEP) (Wilkie, 2002). However, the experimental evidence for this assumption is circumstantial at best and it cannot be dismissed that NH_3 excretion occurs, especially since the gulf toadfish display a blood

side negative TEP in seawater (Evans, 1980). Excretion of NH_3 in contrast to NH_4^+ would not confer an acid-base balance advantage during exposure to hypersalinity but would rather contribute to the acidosis arising from intestinal transport processes. The argument for energy conservation obtained by excretion of NH_3/NH_4^+ rather than the metabolically expensive urea relies on the assumption that the metabolic cost of ammonia excretion is negligible compared to the cost of urea synthesis and excretion. Despite significant progress in the understanding of urea excretion by the gulf toadfish (McDonald et al., 2006) the metabolic cost remains unknown. Similarly, the rapidly developing field of NH_3/NH_4^+ excretion by fish (Nawata et al., 2007; Hung et al., 2007; Nakada et al., 2007) still lacks insight into the energetic driving force and thus the metabolic cost. Unfortunately, urea excretion was not measured as part of the present study, but considering the observed NH_3/NH_4^+ excretion increase, the above discussion is relevant in the case of unaltered or reduced urea excretion and we can infer an apparent impact of intestinal osmoregulatory processes on branchial nitrogenous waste excretion. To our knowledge, this is the first report of increased NH_3/NH_4^+ excretion at the gill being linked to a hyperosmoregulatory challenge. Directly investigating this connection between salinity, acid-base balance, and the mode of nitrogenous waste excretion is an exciting area for future work.

Overview and future directions

It is generally acknowledged that most teleost fish display an acidosis and decreased HCO_3^- equivalents in the blood plasma upon transfer from freshwater to seawater (Wilkes and McMahon, 1986; Nonnotte and Truchot, 1990; Maxime et al., 1991), a

phenomenon also observed in this study following transfer to elevated salinity. Concentrations of Cl^- and Na^+ determined in this study also agree with previous work (Bath and Eddy, 1979; Wilkes and McMahon, 1986; Maxime et al., 1991), with both ions increasing in the plasma over the first 24 h post-transfer, and absorption of Cl^- into the extracellular fluids increasing to a greater extent than Na^+ (Table 2.1, Fig. 2.8). Overall, we conclude that increased intestinal HCO_3^- secretion in elevated salinity in the gulf toadfish creates an acid-base balance disturbance, which is rapidly and almost completely corrected by increased branchial H^+ excretion. These two processes offer an explanation for the commonly observed (transient) acidosis following transfer to hypersalinity. To our knowledge, this is the first demonstration of intestinal transport processes involved in osmoregulation having an impact on acid-base balance, as well as net acid extrusion and NH_3/NH_4^+ excretion at the gill. Our observations add to the accepted role of NaCl excretion at the gill compensating for intestinal NaCl absorption, illustrating how these two organs operate in concert to maintain not only salt and water balance, but also acid-base homeostasis. Increased excretion of NH_3/NH_4^+, in response to the acid-base disturbance in high salinity, suggests an additional and unique connection between osmoregulation and excretion of nitrogenous waste. The role of the kidney on acid-base balance with regards to physiological demands of salinity transfer has previously been investigated and found to be relatively minimal, particularly in aglomerular fish, such as *O. beta*, but it would be worthwhile to perform integrative experiments to clarify how the renal processes might be related to the salinity response observed in this study.

The extent to which intestinal osmoregulation impacts acid-base balance and the importance of other organs, notably the gill, in compensating for acid-base disturbances

in high salinity may be variable among species. Species which naturally see large, rapid salinity fluctuations due to tidal movements would be predicted to have faster, more efficient adjustment mechanisms for osmoregulation and maintenance of acid-base balance when presented with a salinity challenge, and could be promising candidates for future work in this area. However, such species may continuously possess osmoregulatory and acid-base balance mechanisms to accommodate a range of salinities. Fish which rarely see fluctuations in salinity (*i.e.* anadromous species such as many salmonids) may offer insight into the cellular mechanisms regulating water, ion, and acid-base balance, particularly at the transcriptional level, as these mechanisms likely would be recruited during environmental challenges associated with salinity change. Examples of this for gill tissue are ample in the current literature (Marshall and Grosell, 2005).

Chapter 3

Concentration of $MgSO_4$ in the intestinal lumen of *Opsanus beta* limits osmoregulation in response to acute hypersalinity stress

Summary

Marine teleosts constantly lose water to their surrounding environment, a problem exacerbated in fish exposed to salinity higher than normal seawater. Some fish undergo hypersaline exposures in their natural environments, such as short and long-term increases in salinity occurring in small tidal pools and other isolated basins, lakes, or entire estuaries. Regardless of the degree of hypersalinity in the ambient water, intestinal absorption of monovalent ions drives water uptake to compensate for water loss, concentrating impermeable $MgSO_4$ in the lumen. This study considers the potential of luminal [$MgSO_4$] to limit intestinal water absorption, and therefore osmoregulation, in hypersalinity. The overall tolerance and physiological response of toadfish (*Opsanus beta*) to hypersalinity exposure were examined. *In vivo*, fish in hypersaline waters containing artificially low [$MgSO_4$] displayed significantly lower osmolality in both plasma and intestinal fluids, and increased survival at 85 ppt seawater, indicating improved osmoregulatory ability than in fish exposed to hypersalinity with ionic ratios similar to naturally occurring ratios. Intestinal sac preparations revealed that in addition to the osmotic pressure difference across the epithelium, the luminal ionic composition influenced the absorption of Na^+, Cl^-, and water. Hypersalinity exposure increased urine flow rates in fish fitted with ureteral catheters regardless of ionic composition of the ambient seawater, but had no effect on urine osmolality or pH. Overall, concentrated

$MgSO_4$ within the intestinal lumen, rather than renal or branchial factors, is the primary limitation for osmoregulation by toadfish in hypersaline environments.

Background

In marine environments, fish are hypoosmotic to their surroundings and suffer from continual diffusive water loss and influx of salts. The primary solution to this problem is active ingestion of the ambient water (Shehadeh and Gordon, 1969), followed by absorption of salts and water by the gut (Smith, 1930; Skadhauge, 1969; Skadhauge, 1974), with subsequent branchial excretion of monovalent ions and rectal or renal excretion of divalent ions (Beyenbach, 2004; Marshall and Grosell, 2005; Evans et al., 2005), resulting in water gain which balances the diffusive water loss. Maintaining water balance is thus an integrative process requiring the interaction of three osmoregulatory organs of the fish: the gut, gill, and kidney.

While the osmoregulatory mechanisms in both freshwater and marine teleosts have been studied in detail, investigations of hypersaline exposure are relatively rare and none of these studies have investigated limiting factors on survival to high salinities. Some species in which hypersaline osmoregulation has been studied include the sailfin molly (Gonzalez et al., 2005), 'California' Mozambique tilapia (Sardella et al., 2004), gilthead sea bream (Sangiao-Alvarellos et al., 2003), Senegalese sole (Arjona et al., 2007), eel (Maetz and Skadhauge, 1968; Skadhauge, 1969) and gulf toadfish (McDonald and Grosell, 2006). In general, exposure to hypersalinity exacerbates the requirements of the hypo-osmoregulatory system (Skadhauge, 1969); drinking rate,

plasma osmotic pressure, intestinal absorption and plasma concentrations of Na^+ and Cl^-, as well as Na^+-K^+-ATPase activity in the gut and gill, are all greater than observed in seawater.

Survival under conditions of extreme ambient salinity may be limited by each of the three organs involved in osmoregulation by marine teleost fish. First, elevated ambient salinity will challenge fish with increased uphill Na^+ and Cl^- gradients, opposing excretion of these ions across the gill (Chapter 2). Second, three impacts on the intestinal contribution to osmoregulation during hypersaline exposure are likely important in determining tolerance: an increase in drinking rate, increased Na^+ and Cl^- absorption by the epithelium (Grosell et al., 2009a), and higher concentrations of Mg^{2+} and SO_4^{2-} in the intestinal lumen as the absorption of Na^+, Cl^-, and water increases (Chapter 2). At high salinities, as Na^+, Cl^-, and water are absorbed Mg^{2+} and SO_4^{2-} become the dominant electrolytes in the luminal fluids due to the relative impermeability of the epithelium to divalent ions (Hickman, 1968; Shehadeh and Gordon, 1969), potentially limiting water absorption by the intestinal epithelium (Chapter 2). These processes create a scenario in which permeable ions comprise a smaller fraction of the luminal fluid osmotic pressure, and impermeable ions become increasingly concentrated due to higher epithelial water absorption, making uptake of Na^+, Cl^-, and thus water, increasingly difficult. Hypothetically, toadfish should have greater hypersalinity tolerance if the encumbrance imposed by concentrated $MgSO_4$ in the lumen is removed or reduced, as this may enhance the ability of the epithelium to absorb water. Last, while luminal $MgSO_4$ may thus limit tolerance to extremely high salinities, some $MgSO_4$ is inevitably taken up by the intestine and must be excreted, a function of the marine teleost kidney (Hickman and

Trump, 1969). Since marine teleosts are incapable of forming hyperosmotic urine (Hickman and Trump, 1969), an increased demand for renal $MgSO_4$ excretion is likely to result in higher rates of urine flow, and thus water loss, posing another potential limitation for survival in hypersaline environments.

The organism used in the present study, *Opsanus beta* (Goode and Bean) is a subtropical species of toadfish found throughout Biscayne and Florida Bays. These fish are bottom-dwelling (Böhlke and Chaplin, 1993) and inhabit small home ranges, although seasonal habitat shifts are undergone by this species, likely associated with temperature and/or reproduction (Serafy et al., 1997). The distribution of toadfish is correlated with several variables, including depth, temperature, dissolved oxygen, and density of the seagrass *Thalassia* (Sogard et al., 1987; Serafy et al., 1997). Salinity, however, does not appear to be a contributing factor determining abundance and toadfish are naturally exposed to a wide salinity range (Lorenz and Serafy, 2006), with a maximum mean Bay-wide salinity reaching 41.8 ppt seawater (Kelble et al., 2007); toadfish have been observed residing in areas where local mean salinities are as high as 59.3 ppt (Ley et al., 1999). Considering the characteristics of its natural environment, it is perhaps not surprising that *O. beta* is known to maintain normal water and salt balance between 5 and 50 ppt seawater, and can survive for at least 7 days in 70 ppt seawater (McDonald and Grosell, 2006).

In this study we investigated the physiological response of *O. beta* to hypersalinity, with the objective of determining which of the osmoregulatory processes (intestinal water absorption, renal water loss associated with excretion of divalent ions, or branchial extrusion of Na^+ and Cl^- against an increased gradient) limits survival of

toadfish exposed to hypersalinity. While the impact of $MgSO_4$ independent of NaCl has been investigated with respect to kidney function and urine production (McDonald and Walsh, 2007), this is the first study to consider the impact of $MgSO_4$ concentration within the intestinal lumen on water balance. Our hypotheses were that Na^+ and Cl^- extrusion at the gill would be limited by elevated ambient NaCl, and that water absorption by the intestine under hypersaline conditions would be limited by concentrated luminal $MgSO_4$. However, an alternative hypothesis is that in fish exposed to hypersalinity, intestinal uptake of Mg^{2+} and SO_4^{2-} will increase, resulting in increased demand for renal ion excretion and therefore water loss. This third potential limitation on osmoregulation in hypersalinity was also tested as part of the present study.

Materials and Methods

Experimental Animals

Gulf toadfish (*Opsanus beta*) were obtained from local shrimp fishermen and kept in aerated, 62 l tanks receiving a continuous flow of natural filtered seawater from Biscayne Bay (Bear Cut, 34-37 ppt seawater, 22-26°C) with shelters of polyvinyl chloride tubing. Immediately upon receipt, fish were treated with malachite green to remove external parasites (McDonald et al., 2003). Fish were fed to satiation weekly, but food was withheld at least 4 days before experimentation. Fish were kept in the lab and used in experiments according to an approved University of Miami Animal Care Protocol (IACUC #09-001).

Exposure to Hypersalinity

Fish were transferred in groups of 6-8 to hypersaline (50, 60, 70, 77, and 85 ppt salinities) 62 l aerated tanks, with water continuously circulated through a biofilter. Two types of hypersaline waters were made by adding either sea salt (Instant Ocean) or NaCl alone to 35 ppt seawater. The osmolalities of the two hypersaline waters at each salinity varied by no more than 1.7%. The concentrations of Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, Ca^{2+}, K^+, and total CO_2 in these media are given in Table 3.1. After a 24 h exposure period, blood samples were obtained by caudal puncture and immediately centrifuged to isolate the plasma. Toadfish were then euthanized with an overdose of tricaine methanesulfonate (MS-222) anesthetic (2 $g \cdot l^{-1}$) and intestinal fluid samples were collected by dissecting the full length of the intestine between the pyloric and rectal sphincters and draining the luminal fluid into a sample tube. The osmolality and total CO_2 concentrations of the plasma and intestinal fluids were measured immediately after sampling, and samples were frozen at -20˚C for later ion analysis.

Intestinal Sac Preparations

Four sets of experiments using intestinal sac preparations were performed. Each experiment was planned to address a specific aspect of luminal [NaCl] and [$MgSO_4$] effects on salt and water transport across the intestinal epithelium. The ionic composition and osmolalities of the salines used in these experiments are shown in Tables 3.2-3.5.

Intestinal sac preparations were made according to the following protocol, modified from Grosell and Taylor (2007). Anaesthetized (0.5 $g \cdot l^{-1}$ MS-222) fish (35 ppt seawater) were sacrificed by a blow to the head followed by spinal transection, and the

anterior intestinal segment was isolated. A filling catheter of PE50 (I.D. 0.58 mm, O.D. 0.965 mm) tubing, heat flared on one end, was inserted into the anterior end of the intestinal segment and tied in place with 3-0 silk sutures. The tissue was then rinsed with 5 ml of the appropriate luminal saline (see Tables 3.2-3.5) to remove any intestinal contents. The distal end of the tissue segment was then tied closed with 3-0 silk. The resulting sac preparation was filled with luminal saline containing 0.5 μCi ml^{-1} polyethylene glycol ^{14}C-4000 (PEG, specific activity 13.0 mCi g^{-1}), sealed, and the initial full weight of the sac was determined. The 1 ml Hamilton syringe containing the filling saline was weighed before and after filling the sac preparation to determine the exact volume of saline added to the sac, and the saline remaining in the syringe was kept as a reference initial sample of mucosal saline. To ensure that the remaining saline in the syringe was reflective of the saline contained in the sac preparation, saline was flushed back and forth between the sac preparation and the syringe three times prior to sealing the filling catheter. The filled sac was placed in a scintillation vial containing 15 ml serosal saline, which was gassed with 0.3 % CO_2/O_2 mix for at least 30 minutes prior to and throughout the flux period, and an initial 1 ml serosal saline sample was taken. After a 2 h flux period, the sac preparation was removed from the vial and a final 1 ml serosal saline sample was taken. The sac preparation was blotted dry and weighed to determine the final full sac weight. The sac was cut open at the distal sutures and the luminal saline collected in a tube. Subsequently, the sac was cut longitudinally and blotted dry, and the weight of the empty sac including the filling catheter and sutures was determined. The catheter and sutures were removed and the tissue segment was stored overnight at 4˚C to allow the tissue to relax prior to determining the surface area by tracing the spread-out

tissue segment on paper, weighing the traced area, and correcting against the weight of 1 cm^2 paper. Radioactivity of samples of the initial saline and saline collected before and after the flux period were measured using a Packard liquid scintillation analyzer (Tri-Carb 2100TR), following the protocol described in Grosell and Taylor (2007). Initial and final saline samples were also measured for pH, total CO_2, osmolality, and ionic composition as described below.

Experimental Set 1: Luminal salines (Table 3.2) were designed to mimic the ion concentrations measured in the intestinal fluids of fish exposed *in vivo* to seawater (35 ppt) supplemented with sea salt to 70 ppt or supplemented with NaCl alone to 70 ppt (Table 3.6). *In vivo*, the intestinal fluid osmolalities of fish from these two exposure groups were different. Thus, an additional treatment was added in which the osmolality of luminal saline mimicking the intestinal fluids of fish exposed to 70 ppt water supplemented with NaCl (350 mosm kg^{-1}) was adjusted with mannitol to be isoosmotic to the luminal saline mimicking intestinal fluids of fish exposed to 70 ppt water supplemented with sea salt (374 mosm kg^{-1}).

Experimental Set 2: To examine the impact of luminal ionic composition on intestinal fluid absorption, [$MgSO_4$] and [NaCl] of the luminal saline were adjusted in opposing directions, so the saline with the highest [NaCl] (150 mM) had the lowest [$MgSO_4$] (59.5 mM) and vice versa, maintaining the same osmolality in all treatments (Table 3.3).

Experimental Set 3: Next, the impact of intestinal fluid composition on the ability of the tissue to absorb fluid against an osmotic gradient was determined.

Table 3.1. Ionic composition (mM) and tCO_2 of 50, 60, 70, 77, and 85 ppt seawater adjusted from 35 ppt with sea salt or adjusted from 35 ppt with NaCl only. The 35 ppt seawater reference values are from earlier studies (Chapter 2).

	35 ppt	50 ppt		60 ppt		70 ppt		77 ppt		85 ppt	
		Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl
Na^+	454	526	557	566	720	710	991	914	949	978	1075
Cl^-	440	551	555	577	707	764	928	956	903	1063	1048
Mg^{2+}	46	63	49	76	50	90	57	105	50	118	53
SO_4^{2-}	24	34	28	42	27	38	23	50	27	49	30
Ca^{2+}	--	12	9	14	10	15	11	20	10	21	11
K^+	--	12	10	15	10	18	11	22	10	26	11
tCO_2	--	3	2	4	2	3	3	6	3	3	2

Table 3.2. Composition of serosal and luminal salines for experimental set #1. All concentrations are expressed in mM; osmolality in mosm kg^{-1}. Serosal saline was gassed with 0.3% CO_2/O_2 mix prior to and throughout the 2 h flux period.

	Serosal	Luminal		
		70 ppt Sea salt	70 ppt NaCl	NaCl Isoosmotic
NaCl	177.0	31.0	60.0	60.0
$MgSO_4 \cdot 7H_2O$	0.9	228.0	174.0	174.0
$MgCl_2 \cdot 6H_2O$	--	12.0	6.0	6.0
KCl	3.0	3.0	3.0	3.0
$CaCl_2 \cdot 2H_2O$	1.0	6.0	3.0	3.0
$NaHCO_3$	5.0	1.0	1.0	1.0
Na_2HPO_4	0.5	--	--	--
KH_2PO4	0.5	--	--	--
Hepes (free acid)	5.5	--	--	--
Hepes (Na salt)	5.5	--	--	--
Urea	4.5	--	--	--
Glucose	5.0	--	--	--
Mannitol	--	--	--	13.0
mOsm	372	374	350	374

Table 3.3. Composition of serosal and luminal salines for experimental set #2. All concentrations are expressed in mM; osmolality in mosm kg^{-1}. Serosal saline was gassed with 0.3% CO_2/O_2 mix prior to and throughout the 2 h flux period.

	Serosal	Luminal			
		60 mM	90 mM	120 mM	150 mM
NaCl	180.3	60.0	90.0	120.0	150.0
$MgSO_4 \cdot 7H_2O$	0.9	212.0	152.3	106.3	59.5
$MgCl_2 \cdot 6H_2O$	--	6.0	6.0	6.0	6.0
KCl	3.0	3.0	3.0	3.0	3.0
$CaCl_2 \cdot 2H_2O$	1.0	3.0	3.0	3.0	3.0
$NaHCO_3$	5.0	1.0	1.0	1.0	1.0
Na_2HPO_4	0.5	--	--	--	--
KH_2PO4	0.5	--	--	--	--
Hepes (free acid)	5.5	--	--	--	--
Hepes (Na salt)	5.5	--	--	--	--
Urea	4.5	--	--	--	--
Glucose	5.0	--	--	--	--
mOsm	372.5	373	372	373	376

Table 3.4. Composition of serosal and luminal salines for experimental set #3. All concentrations are expressed in mM; osmolality in mosm kg^{-1}. Serosal saline was gassed with 0.3% CO_2/O_2 mix prior to and throughout the 2 h flux period.

	Serosal	Luminal							
		Low [NaCl]				High [NaCl]			
NaCl	151.0	90.0				150.0			
$MgSO_4 \cdot 7H_2O$	0.9	95.1				1.0			
$MgCl_2 \cdot 6H_2O$	--	6.0				6.0			
KCl	3.0	3.0				3.0			
$CaCl_2 \cdot 2H_2O$	1.0	3.0				3.0			
$NaHCO_3$	5.0	1.0				1.0			
Na_2HPO_4	0.5	--				--			
KH_2PO4	0.5	--				--			
Hepes (free acid)	5.5	--				--			
Hepes (Na salt)	5.5	--				--			
Urea	4.5	--				--			
Glucose	5.0	--				--			
Mannitol	--	--	20.7	39.4	57.1	--	16.2	35.7	55.6
mOsm	315	301	321	339	357	305	320	339	361

Table 3.5. Composition of serosal and luminal salines for experimental set #4. All concentrations are expressed in mM; osmolality in mosm kg^{-1}. Serosal saline was gassed with 0.3% CO_2/O_2 mix prior to and throughout the 2 h flux period.

	Serosal	Luminal	
		35 ppt *in vivo*	+60 mM $MgSO_4$
NaCl	151.0	77.8	77.8
$MgSO_4 \cdot 7H_2O$	0.9	70.0	130.0
$MgCl_2 \cdot 6H_2O$	--	30.0	30.0
KCl	3.0	2.0	2.0
$CaCl_2 \cdot 2H_2O$	1.0	2.0	2.0
$NaHCO_3$	5.0	1.0	1.0
Na_2HPO_4	0.5	--	--
KH_2PO4	0.5	--	--
Hepes (free acid)	5.5	--	--
Hepes (Na salt)	5.5	--	--
Urea	4.5	--	--
Glucose	5.0	--	--
mOsm	309	310	369

Two treatments were utilized, one with high [NaCl] (150 mM) and only 1 mM $MgSO_4$, and the other with low [NaCl] (90 mM) and a roughly equal concentration of $MgSO_4$ (95.1 mM). The luminal saline value of 150 mM Na^+ in the high NaCl treatment exceeded physiological levels of Na^+ in the intestinal lumen, while the low NaCl value of 90 mM was closer to the observed luminal concentrations *in vivo* in the present study as well as previous observations (McDonald and Grosell, 2006). Both treatments were adjusted with mannitol from 300 mosm kg^{-1} to approximately 320, 340, and 360 mosm kg^{-1} (Table 3.4).

Experimental Set 4: Finally, the impact of elevated luminal $MgSO_4$ and the associated osmotic pressure on fluid absorption was quantified. Two treatments were used to determine the effect of [$MgSO_4$] and osmolality differences, independent of [NaCl]. The first treatment approximated the ionic composition of luminal fluids of fish in 35 ppt seawater (Taylor and Grosell, 2006). The second luminal saline was identical to the first, but with an additional 60 mM $MgSO_4$ (Table 3.5), allowing for the highest possible [$MgSO_4$] without exceeding luminal osmotic pressure that can be reasonably tolerated *in vivo* (corresponding to ~65 ppt, see Fig. 3.1).

Ureteral catheters

Fish were anesthetized in 0.5 g MS-222 l^{-1} seawater and fitted with ureteral catheters, as described by McDonald *et al.* (2003). PE10 tubing (I.D. 0.28 mm, O.D. 0.61 mm) was filled with 150 mM NaCl solution (colored by adding 3-4 drops of food coloring to a 15 ml aliquot), inserted into the urogenital papilla and advanced dorsally. The catheter was tied to the papilla with 3-0 silk ligatures. After insertion of the catheter, a ventral incision

was made anterior to the rectum and each of the two urinary bladders was ligated as close as possible to the ureter end, thereby bypassing both bladders and resulting in collected urine indicating kidney function only. Oxytetracycline powder was dusted into the body cavity to prevent infection of the wound before closing with running 3-0 sutures. A PE60 (I.D. 0.76 mm, O.D. 1.22 mm) sleeve, heat-flared at each end, was placed on the catheter close to the papilla and joined to the PE10 catheter with glue (Permabond). The sleeve was then sutured to the body below the caudal fin to anchor the catheter.

Catheterized fish were weighed (0.084±0.006 kg) and placed in individual, aerated flux chambers of approx. 2-4 l with 35 ppt seawater and allowed to recover overnight. The ends of the catheters were placed such that urine drained from the catheterized fish into pre-weighed 2 ml sample tubes covered in Parafilm, located approximately 5 cm below the water levels in the flux boxes. Urine and water samples were collected every 12 h for 72 h and the samples were measured immediately upon collection for mass, osmolality, total CO_2 and pH, and stored at -20˚C for later ion analysis.

Analytical Techniques and Calculations

Urine, intestinal fluids, plasma, and saline samples from the experiments described above were all analyzed using the same methods. Osmolality was measured using a vapor pressure osmometer (Wescor 5520), total CO_2 (tCO_2) was measured with a Corning 965 carbon dioxide analyzer, and pH was determined using a microelectrode (Accumet 13-620-96 Fisher Scientific) coupled to a Radiometer pH meter (PHM201). Fluids were analyzed for Cl^- and SO_4^{2-} by anion chromatography (Dionex 120), and for Na^+, Mg^{2+},

K^+, and Ca^{2+} with fast sequential flame atomic absorption spectrometry (Varian 220) using an air/acetylene flame. Base equivalents were calculated from tCO_2 and pH measurements using the Henderson-Hasselbalch equation, as described in Chapter 2.

Ion flux across intestinal sac preparations was calculated as the difference between the initial and final amounts of ion (μmol) in the lumen per cm^2 tissue per hour. ^{14}C PEG-4000 was used as a tracer to determine water movement across the tissue. Water flux was calculated as described by Grosell and Taylor (2007), as the difference in concentration of ^{14}C PEG between initial and final luminal samples per cm^2 tissue per hour. Briefly, the initial volume was calculated as the volume of saline used to fill the sac preparation, corrected for dilution of the filling saline by mixing with fluid contained within the lumen during the filling process. The final volume was calculated as the concentration ratio of ^{14}C PEG of the initial saline and the final saline, multiplied by the initial volume. All radioactivity measurements were corrected for instrument background. Urinary excretion of ions was calculated as the concentration of the ion (mM) in the urine multiplied by the urinary flow rate (ml kg^{-1} h^{-1}).

Statistics

All values are given as mean ± s.e.m. Differences between means were considered significant when $P<0.05$. Differences within treatments in *in vivo* exposure experimental data were examined across salinities using one-way ANOVA, followed by Bonferroni corrected t-tests for normally distributed data. Data that were not normally distributed were examined using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method. Differences between treatments were assessed using Student's t-tests.

Differences between intestinal sac preparation treatments were examined using one-way ANOVA, followed by Bonferroni corrected t-tests for normally distributed data, or Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method for data that was not normally distributed. Renal data was examined for time and treatment effect independently, using one-way ANOVA. Data that were not normally distributed were assessed using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method.

Results

Exposure to Hypersalinity

The osmolality of plasma and intestinal fluids of fish exposed to hypersalinity adjusted from 35 ppt seawater with either sea salt or NaCl significantly increased with increasing salinity (Fig. 3.1). At salinities of 70 ppt and above, fish in the low [$MgSO_4$] treatment had significantly lower osmolality in both plasma and intestinal fluids than fish exposed to hypersaline water of the same osmolalities but with standard seawater ionic ratios. In addition, the osmolalities of the intestinal fluids and plasma were strongly correlated (Fig. 3.2). Fish exposed to 85 ppt water experienced high mortality rates and data from surviving fish are not included in Fig. 3.1. Interestingly, mortality was greater in fish exposed to 85 ppt water adjusted with sea salt (100%) than with NaCl (75%). Precipitates were observed in the intestinal fluids of most fish *in vivo*, but no attempt was made to quantify any aspect of intestinal $CaCO_3$ precipitation.

The ionic compositions and total CO_2 of intestinal fluids and blood plasma of fish exposed to hypersalinity are shown in Tables 3.6 and 3.7, respectively. Ionic concentrations of intestinal fluids could not be measured in the sea salt treatment at 77

and 85 ppt salinities due to small sample volumes. However, in the intestinal fluids for which analyses could be completed, Na^+ was consistently elevated in samples from the NaCl treatment compared to samples from fish in seawater supplemented with sea salt. Similarly, Mg^{2+}, SO_4^{2-}, and Ca^{2+} concentrations all were greater in the sea salt (high $MgSO_4$) group than the NaCl group at 60 ppt salinity, with this trend continuing at the higher salinities. There were few differences between treatments in the concentration of ions in the plasma, although Ca^{2+} and K^+ were less concentrated in the plasma of fish exposed to NaCl-adjusted seawater, particularly at 70 and 77 ppt salinities.

Intestinal Sac Preparations

The first set of experiments using intestinal sac preparations was designed to mimic the *in vivo* exposures; the luminal salines (Table 3.2) were created based on the observed ion concentrations of intestinal fluids from fish exposed to each of the 70 ppt waters (Table 3.6) with the exception of HCO_3^-, which was maintained at a low concentration to avoid precipitation. A clear difference between the compositions of the two luminal 70 ppt salines, reflecting the *in vivo* observations, was a lower osmolality in the intestinal fluid of fish exposed to the NaCl treatment (Table 3.2, Fig. 3.1). The substantial water loss observed under conditions mimicking *in vivo* fluid chemistry of 70 ppt sea salt exposed fish was abolished in preparations exposed to salines mimicking the fluid chemistry of fish exposed to 70 ppt water adjusted with NaCl, which had lower luminal $MgSO_4$ (Fig. 3.3A). The difference in water flux rates between these two groups was only partly due to osmotic pressure differences, as evident from continued, although reduced, water loss even when the osmolality of salines mimicking fluid chemistry from fish exposed to 70

ppt water supplemented with NaCl was equalized to that of the 70 ppt sea salt group (Fig. 3.3A). We therefore attribute the significant difference in water flux between the *in vivo*-like treatments to a combined effect of the difference in osmolality and the ionic compositions of the salines. A similar pattern was observed for Ca^{2+} flux, while Mg^{2+}, SO_4^{2-}, and K^+ fluxes were the same in all treatments (Table 3.8).

Water and most ion fluxes differed significantly among treatments of increasing [NaCl] and decreasing [$MgSO_4$] (Fig 3.4). The absorption of water, Na^+, and Cl^- by the intestinal epithelium increased as [NaCl] increased and [$MgSO_4$] decreased (Fig. 3.4A, B). In the treatments with higher [NaCl] and lower [$MgSO_4$], secretion of Mg^{2+} and SO_4^{2-} appeared to be reduced, with mean fluxes near zero (Fig. 3.4C), and the secretion of HCO_3^- was reduced (Fig. 3.4E). Net K^+ absorption is significantly elevated by increasing [NaCl], while Ca^{2+} fluxes were unaffected by luminal chemistry in these experiments (Fig. 3.4D)

Water and ion fluxes at two different NaCl and $MgSO_4$ concentrations and varying luminal osmolality are shown in Figure 3.5. As expected, absorption of water across the tissue decreased with increasing luminal osmolality regardless of luminal NaCl and $MgSO_4$ concentrations (Fig. 3.5A). Preparations exposed to the luminal saline dominated by NaCl, with only 1 mM $MgSO_4$, had significantly higher water flux than the treatment where these salts were of approximately equal concentrations. Increasing luminal osmolality did not affect absorption of Na^+, but Cl^- absorption increased with osmolality and was significantly higher in both treatment groups at 360 mosm kg^{-1} when compared to 300 mosm kg^{-1} (Fig. 3.5B).

Overall absorption of Na^+ was significantly greater in the high [NaCl] treatment. Similarly, the secretion of SO_4^{2-} was significantly higher in the low [NaCl] treatment, which had a greater [$MgSO_4$] (Fig. 3.5C). There was no change in the flux of Mg^{2+}, SO_4^{2-}, K^+, or Ca^{2+} with respect to osmolality (Fig. 3.5C and D). However, intestinal HCO_3^- secretion increased significantly with osmotic pressure in the low [NaCl] treatment (Fig. 3.5E). Lastly, the addition of 60 mM $MgSO_4$ to a mucosal saline mimicking intestinal fluids observed in toadfish exposed to 35 ppt seawater prevented water absorption across the tissue (Fig. 3.6A) without affecting any of the measured ion fluxes (Fig. 3.6B-E).

Renal Contribution

Overall, ureteral urine flow rates (Fig. 3.7A) in both hypersaline treatments were significantly higher than the 35 ppt seawater control with the 70 ppt NaCl group having the highest rates. The urine flow rates in the 70 ppt NaCl group were greater than the control at 72 h. The osmolality was greater in the NaCl-adjusted 70 ppt treatment at 36 h (Fig. 3.7B). There was no difference in the pH of the urine in any treatment at any time point (Table 3.9). Over the 72 h flux period, there were no differences in the urinary excretion of either Na^+ or Cl^- between the 70 ppt treatments and the 35 ppt seawater control (Fig. 3.8A, B). In accord with differences in ambient Mg^{2+} and SO_4^{2-} there was a notable impact on Mg^{2+} and SO_4^{2-} excretion (Fig. 3.8C, D). At the later time points, there was a pronounced elevation of Mg^{2+} and SO_4^{2-} excretion in the NaCl-adjusted treatment. Similarly, ion concentrations were unchanged in urine, except for Mg^{2+} which was significantly higher in both high salinity treatments (Table 3.10).

Figure 3.1. Osmolality (mosm kg^{-1}) of (A) plasma (n=6-8), and (B) intestinal fluids (n=4-8) from toadfish exposed to 50, 60, 70, and 77 ppt seawater supplemented with sea salt or seawater supplemented with NaCl only. Dashed line indicates average osmotic pressure measured in toadfish acclimated to 35 ppt seawater (Taylor and Grosell, 2006). An asterisk (*) indicates significant difference between treatments at the same salinity. Differences across salinities were significant in all treatments; differences among individual means are indicated by differing letters, shared letters indicate no difference.

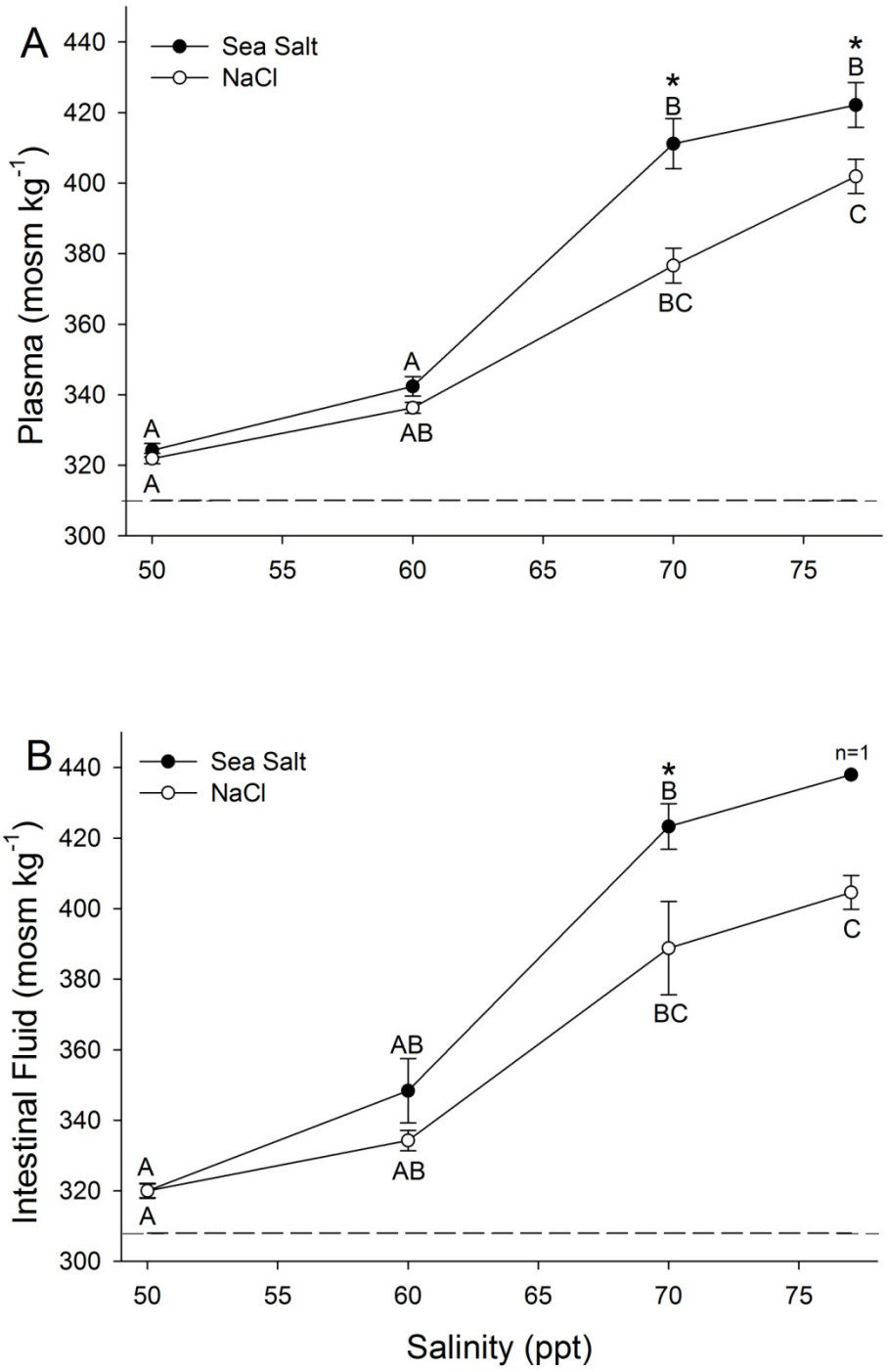


Figure 3.2. Osmolality (mosm kg^{-1}) of plasma as a function of intestinal fluid osmotic pressure from toadfish exposed to 50, 60, 70, 77, and 85 ppt seawater supplemented with sea salt (n=21) or seawater supplemented with NaCl only (n=23). y = 1.034x - 9.377

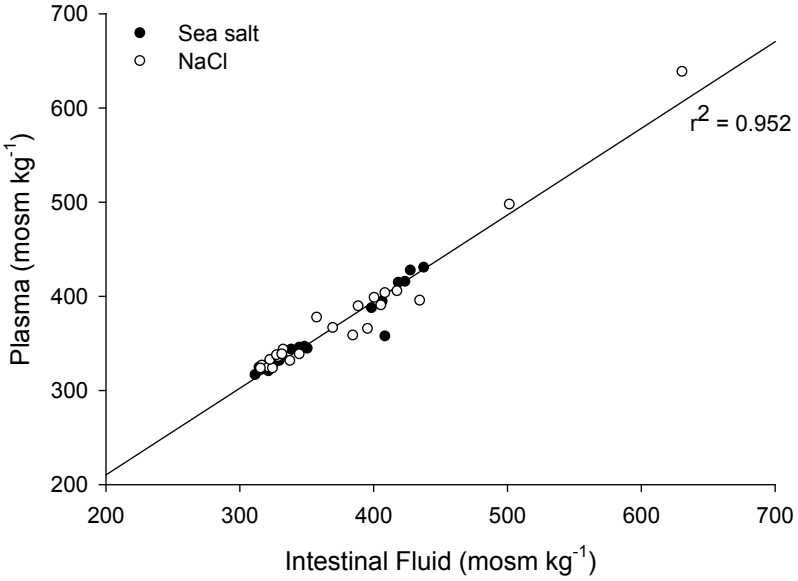


Table 3.6. Ionic composition of intestinal fluids in toadfish exposed for 24 h to 50, 60, 70, 77, and 85 ppt seawater; adjusted from 35 ppt seawater with sea salt or adjusted from 35 ppt seawater with NaCl only. The 35 ppt seawater reference values are from earlier studies. An asterisk (*) indicates significant difference between treatments at the same salinity.

	35 ppt	50 ppt		60 ppt		70 ppt		77 ppt		85 ppt	
		Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl
Na^+	29.8±6.07	22.2±2.16	47.5±6.73*	21.6±1.32	29.7±3.51*	31.2±4.90	67.4±12.90*	--	43.5±3.47	--	226.4±30.27
Cl^-	51.9±4.82	81.4±8.77	77.9±4.99	96.7±12.47	55.3±3.13*	70.1±3.91	79.3±11.69	--	48.8±0.49	--	148.3±54.78
Mg^{2+}	158.5±11.69	185.5±15.80	169.8±23.09	205.6±12.13	135.4±6.49*	207.5±19.02	155.1±19.18	--	235.1±0.65	--	144.5
SO_4^{2-}	97.1±6.44	131.4±10.98	116.9±14.41	151.1±7.12	92.8±5.69*	163.1±17.19	132.2±10.41	--	212.6±0.60	--	166.7
Ca^{2+}	1.8±0.65	6.1±0.51	5.5±0.78	6.8±0.66	3.7±0.60*	6.1±0.85	3.1±0.21*	--	3.3±1.01	--	2.1±0.07
K^+	1.7±0.43	1.5±0.17	2.4±0.37*	2.0±0.43	2.3±0.36	3.2±0.85	2.7±0.25	--	2.6±0.54	--	4.4
tCO_2	62.4±4.03	85.5±3.95	77.8±7.49	71.1±5.64	78.8±4.15	91.2±5.71	68.4±5.89*	45.0±12.10	77.1±8.27	--	50.2±32.94

Table 3.7. Ionic composition of plasma in toadfish acutely exposed for 24 h to 50, 60, 70, 77, and 85 ppt seawater; adjusted from 35 ppt seawater with sea salt or adjusted from 35 ppt seawater with NaCl only. The 35 ppt seawater reference values are from earlier studies (Taylor and Grosell, 2006). An asterisk (*) indicates significant difference between treatments at the same salinity.

	35 ppt	50 ppt		60 ppt		70 ppt		77 ppt		85 ppt	
		Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl	Sea salt	NaCl
Na^+	143.6±3.0	149.4±1.97	152.4±4.45	163.5±5.90	160.6±6.37	185.0±4.97	166.0±5.71*	200.7±5.56	187.9±5.63	--	225.0±32.96
Cl^-	118.5±1.4	109.9±1.70	116.4±2.17*	131.9±2.85	130.1±5.27	149.4±5.25	142.0±5.72	182.6±5.27	167.5±4.96	--	217.8±27.62
Mg^{2+}	--	1.0±0.05	0.9±0.03	1.0±0.08	0.9±0.06	1.2±0.04	0.9±0.06*	1.4±0.03	1.1±0.10	--	2.0±0.48
Ca^{2+}	2.0±0.1	3.4±0.71	2.9±0.40	2.5±0.07	2.2±0.12	2.5±0.06	2.3±0.09	2.6±0.12	2.2±0.10*	--	2.0±0.35
K^+	2.5±0.2	4.6±0.25	3.3±0.10*	5.6±0.91	4.0±0.37	5.5±0.33	4.3±0.84*	5.7±0.22	3.3±0.10*	--	5.3±1.27
tCO_2	5.2±0.6	7.4±0.43	7.6±0.33	5.6±0.21	5.4±0.46	5.1±0.38	5.7±0.19	5.2±0.37	7.7±0.31*	--	8.7±2.06

Table 3.8. Mg^{2+}, SO_4^{2-}, K^+ and Ca^{2+} flux (μmol cm^{-2} h^{-1}) across the intestinal epithelium of intestinal sac preparations exposed to luminal fluid mimicking *in vivo* measurements of intestinal fluids in toadfish exposed to 70 ppt water adjusted with sea salt or adjusted with NaCl only and saline mimicking conditions from 70 ppt water adjusted with NaCl only but with osmolality elevated to that of the 70 ppt group (NaCl adjusted) (see Table 3.2). Positive values indicate absorption and negative values indicate secretion. Asterisk (*) indicates significant difference between treatments.

	70 ppt Sea salt	70 ppt NaCl	NaCl adjusted
Mg^{2+}	-1.9±1.33	-2.1±0.86	-2.2±0.38
SO_4^{2-}	-2.3±0.86	-3.3±0.73	-2.7±0.35
Ca^{2+}	-0.1±0.03	-0.0±0.03	-0.0±0.02*
K^+	-0.1±0.08	-0.0±0.02	-0.0±0.03

Figure 3.3. A: Water flux, B: flux of Na^+ and Cl^- (µmol cm^{-2} h^{-1}), and C: secretion of base equivalents (µeqv cm^{-2} h^{-1}) across intestinal sac preparations exposed to luminal salines mimicking *in vivo* measurements of intestinal fluids in toadfish exposed to 70 ppt water adjusted from seawater with sea salt (n=10), adjusted from seawater with NaCl only (n=8-10), and saline mimicking intestinal fluids from fish exposed to 70 ppt water adjusted with NaCl only with osmolality elevated using mannitol to equal the osmolality of the 70 ppt group (NaCl isoosmotic) (n=10). Positive values indicate absorption (transport from the lumen) and negative values indicate secretion (transport into the lumen). Luminal salines are isoosmotic to serosal saline, except for the 70 ppt NaCl treatment (see Table 2 for saline composition).

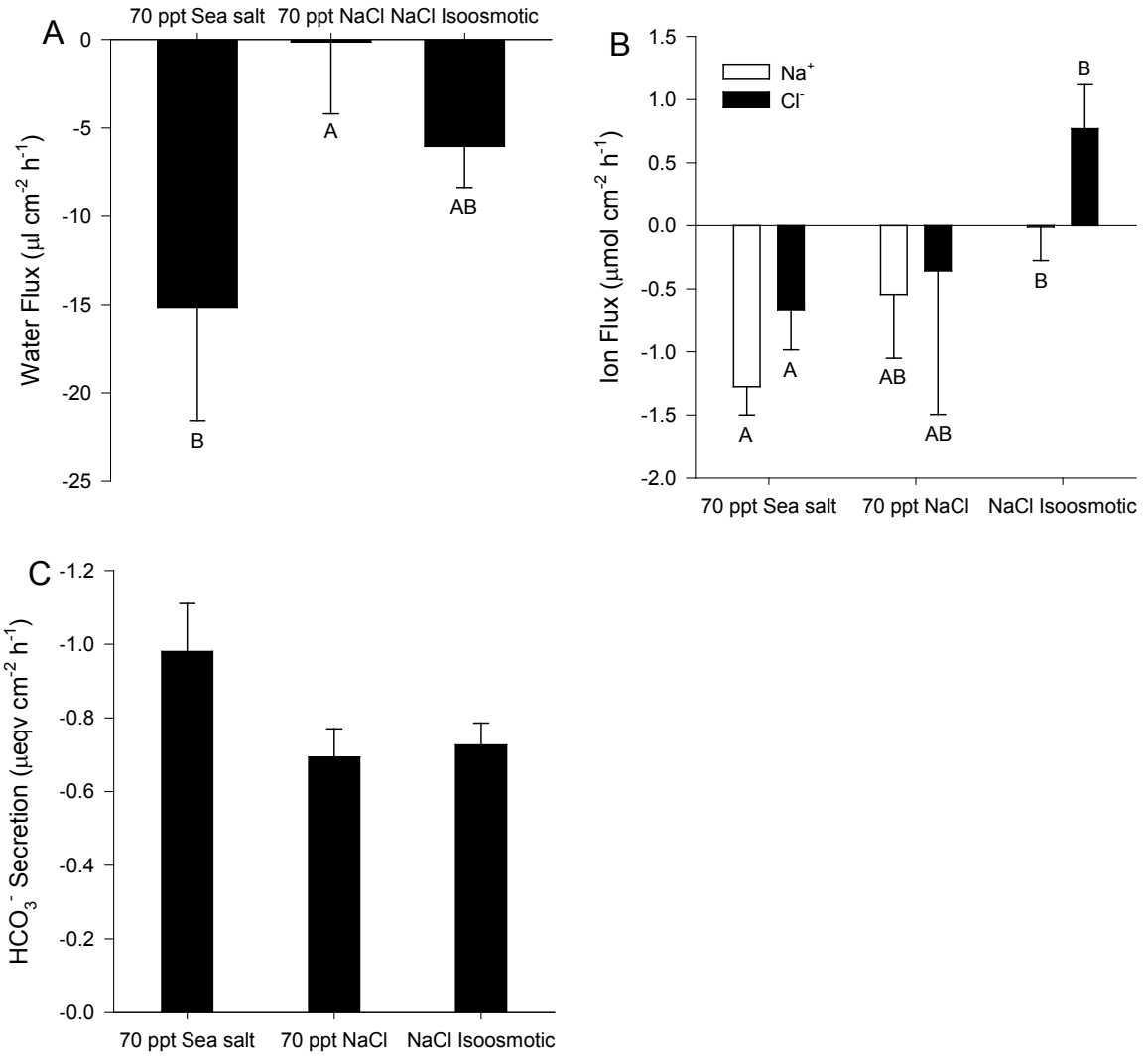


Figure 3.4. Flux of A:water (n=7-8), B: Na^+ and Cl^- (n=6-8), and C: Mg^{2+} and SO_4^{2-} (µmol cm^{-2} h^{-1}) (n=6-8) from intestinal sac preparations exposed to luminal salines varying in [$MgSO_4$] and [NaCl] (mM). Secretion of D: K^+ and Ca^{2+} (µmol cm^{-2} h^{-1}) (n=6-8), and E: base equivalents (µeqv cm^{-2} h^{-1}) (n=5-7) from intestinal sac preparations exposed to luminal salines varying in [$MgSO_4$] and [NaCl] (mM) (see Table 3 for saline composition). Positive values indicate absorption and negative values indicate secretion. Observations marked with different letters (A, B, C) represent significant differences between those treatments; shared letters indicate no difference.

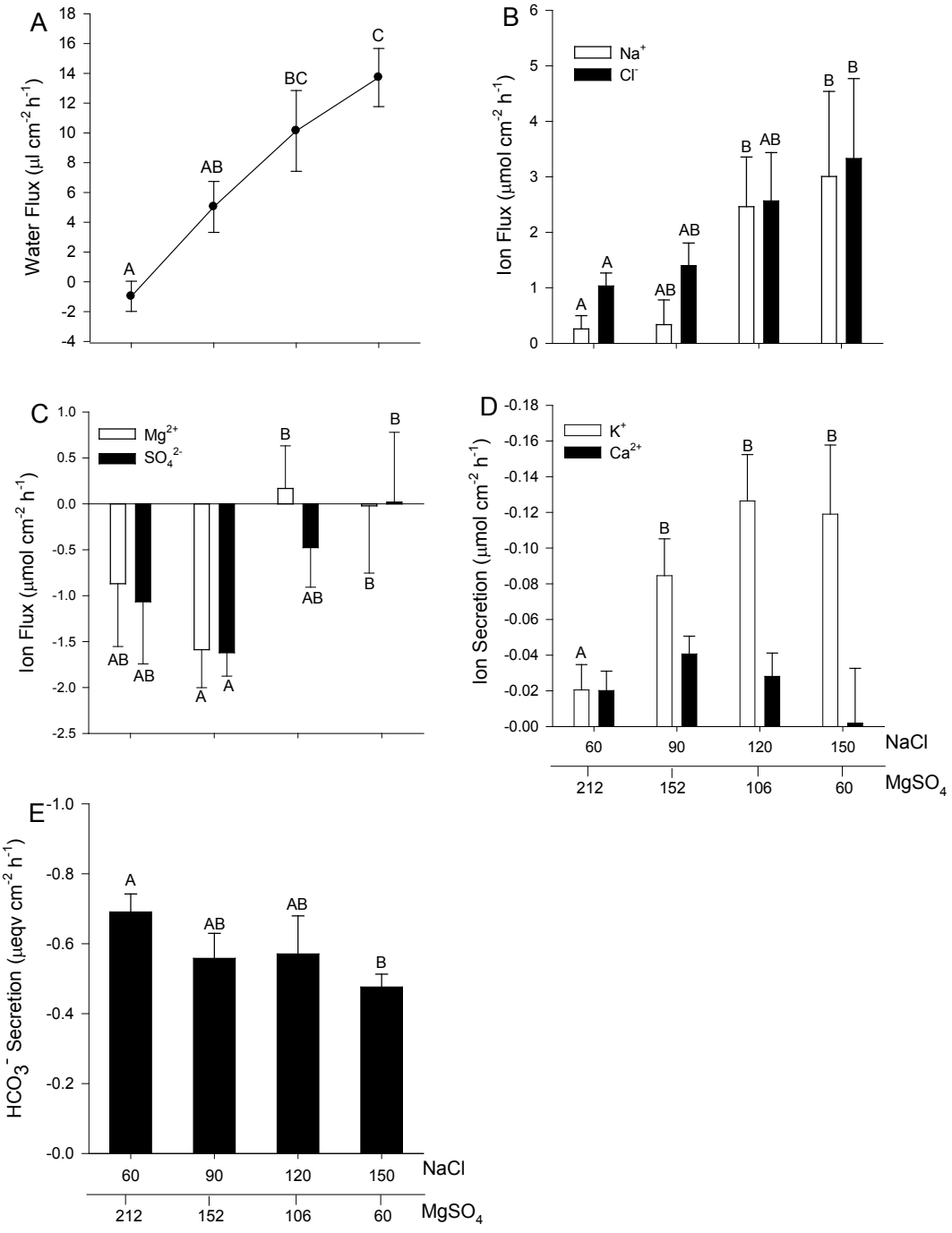


Figure 3.5. A: Water flux (µl cm^{-2} h^{-1}) (n=7-8) of intestinal sac preparations exposed to two luminal salines, each of which has been adjusted with mannitol to approximately 300, 320, 340, and 360 mosm kg^{-1}. High luminal [NaCl] (white) refers to treatment with 150 mM NaCl and 1 mM $MgSO_4$; low luminal [NaCl] (grey) refers to treatment with 90 mM NaCl and 95.1 mM $MgSO_4$ (see Table 4). Positive values indicate absorption and negative values indicate secretion. The point at which the regression lines cross the reference line (dotted) X-axis indicates the shift from water absorption to water loss by the epithelium. Flux of B: Na^+ (open bars) and Cl^- (hatched bars) (n=6-8), C: Mg^{2+} (open bars) and SO_4^{2-} (hatched bars) (n=7-8), and secretion of D: K^+ (open bars) and Ca^{2+} (hatched bars) (µmol cm^{-2} h^{-1}) (n=7), and E: base equivalents (µeqv cm^{-2} h^{-1}) (n=6-8) across intestinal epithelium sac preparations exposed to luminal salines varying in [$MgSO_4$] and [NaCl] (see Table 4 for saline composition). An asterisk (*) indicates significant difference from 300 mosm kg^{-1} treatment. Flux of Na^+, SO_4^{2-}, and K^+ is significantly different between the low [NaCl] treatment and high [NaCl] treatment using ANOVA, but there were no significant differences between individual means.

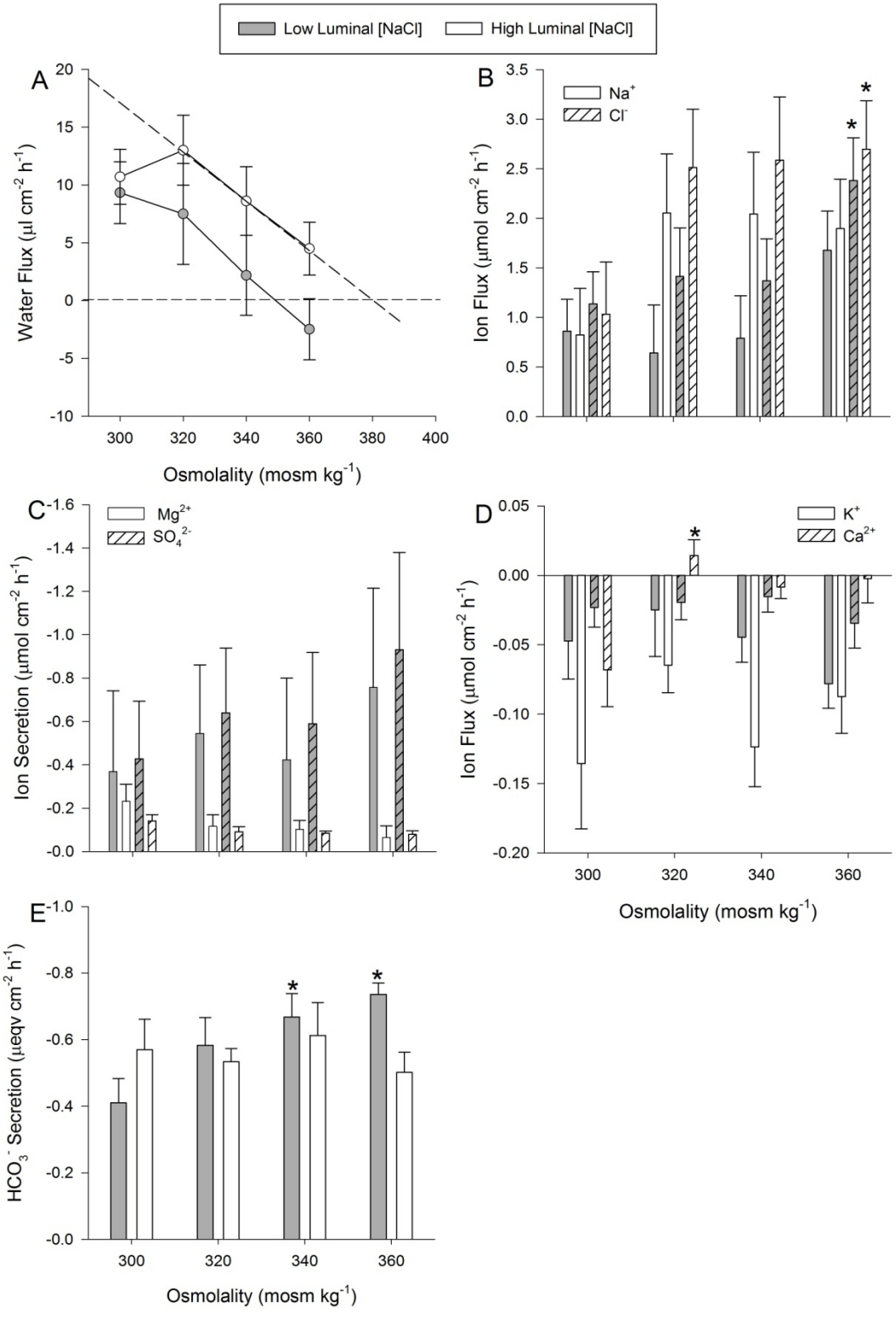

Low Luminal [NaCl]
High Luminal [NaCl]
A
Water Flux (µl cm-2 h-1)
Osmolality (mosm kg-1)
B
Na+
Cl-
Ion Flux (µmol cm-2 h-1)
C
Mg2+
SO4 2-
Ion Secretion (µmol cm-2 h-1)
D
K+
Ca2+
Ion Flux (µmol cm-2 h-1)
Osmolality (mosm kg-1)
E
HCO3 - Secretion (µeqv cm-2 h-1)
Osmolality (mosm kg-1)

Figure 3.6. Flux of A: water (n=7-8), B: Na^+ and Cl^- (n=5-7), C: Mg^{2+} and SO_4^{2-} (n=5-7), D: K^+ and Ca^{2+} (µmol cm^{-2} h^{-1}) (n=4-7), and E: secretion of base equivalents (µeqv cm^{-2} h^{-1}) (n=6-8) across intestinal sac preparations exposed to luminal saline mimicking intestinal fluids of toadfish in 35 ppt seawater, and an identical saline to which 60 mM $MgSO_4$ has been added (see Table 3.5 for saline composition). Positive values indicate absorption and negative values indicate secretion. An asterisk (*) indicates a significant difference between treatments.

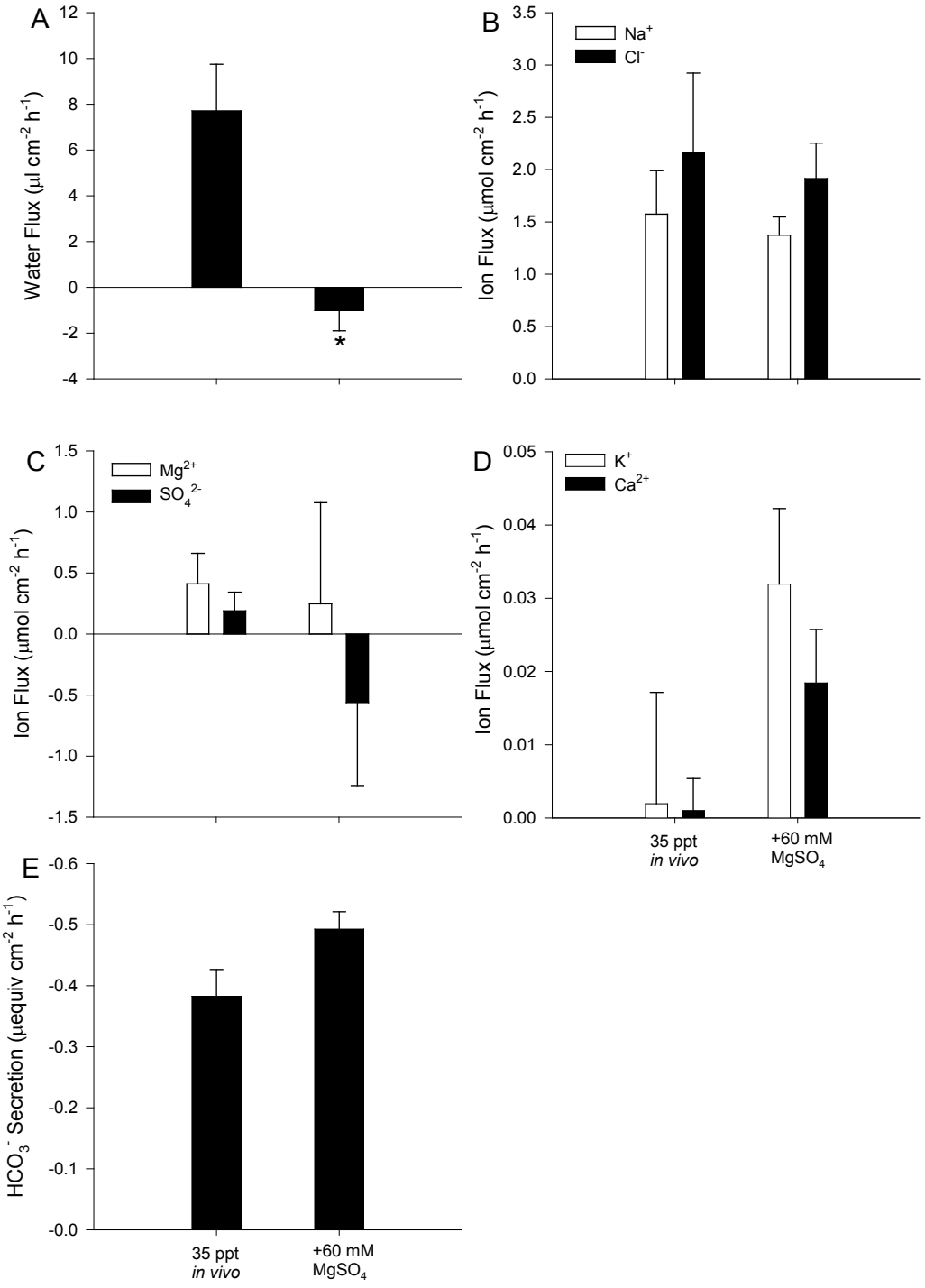


Figure 3.7. A: Urine flow rate (ml kg^{-1} h^{-1}) of gulf toadfish and B: Osmolality (mosm kg^{-1}) of urine collected from gulf toadfish fitted with ureteral catheters exposed to 70 ppt water adjusted from 35 ppt seawater with sea salt or adjusted with NaCl only (n=4-9). The dashed line indicates the transition from control (mean of the 12 and 24 h values) to treatment values. An asterisk (*) indicates difference from 35 ppt seawater at that time point is significant. No differences were observed with respect to time or between high salinity treatments.

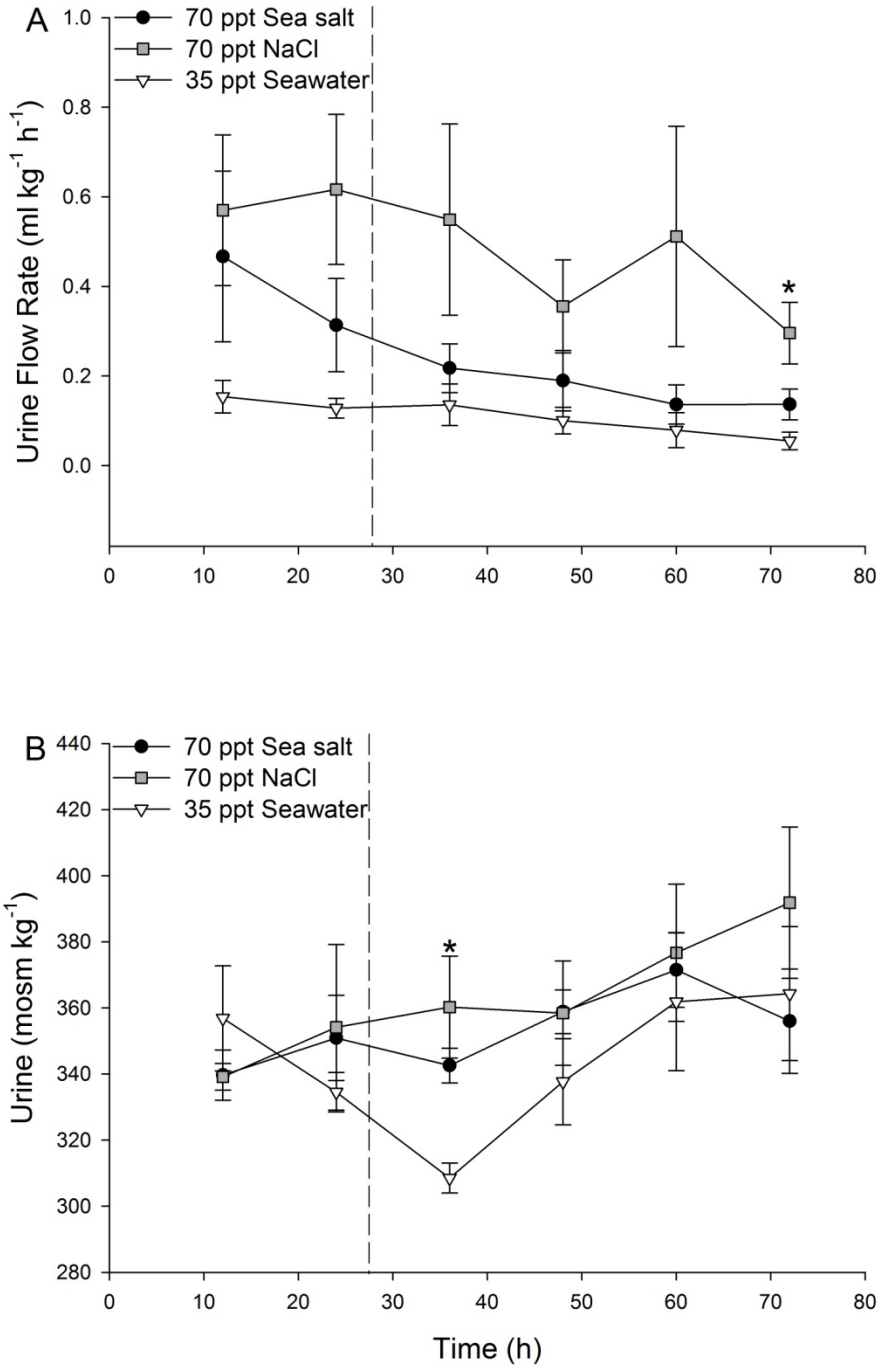


Figure 3.8. Urinary excretion rate (µmol kg^{-1} h^{-1}) of A: Na^{+}, B: Cl^{-}, C: Mg^{+2}, and D: SO_4^{-2} by gulf toadfish fitted with ureteral catheters exposed to 70 ppt water adjusted from seawater with sea salt or adjusted with NaCl only (n=3-6). The dashed line indicates the transition from control (mean of the 12 and 24 h values) to treatment values. * indicates difference from the 35 ppt seawater at that time point is significant. $ indicates significant difference between treatment groups. No differences due to time were observed in any treatment for any ion.

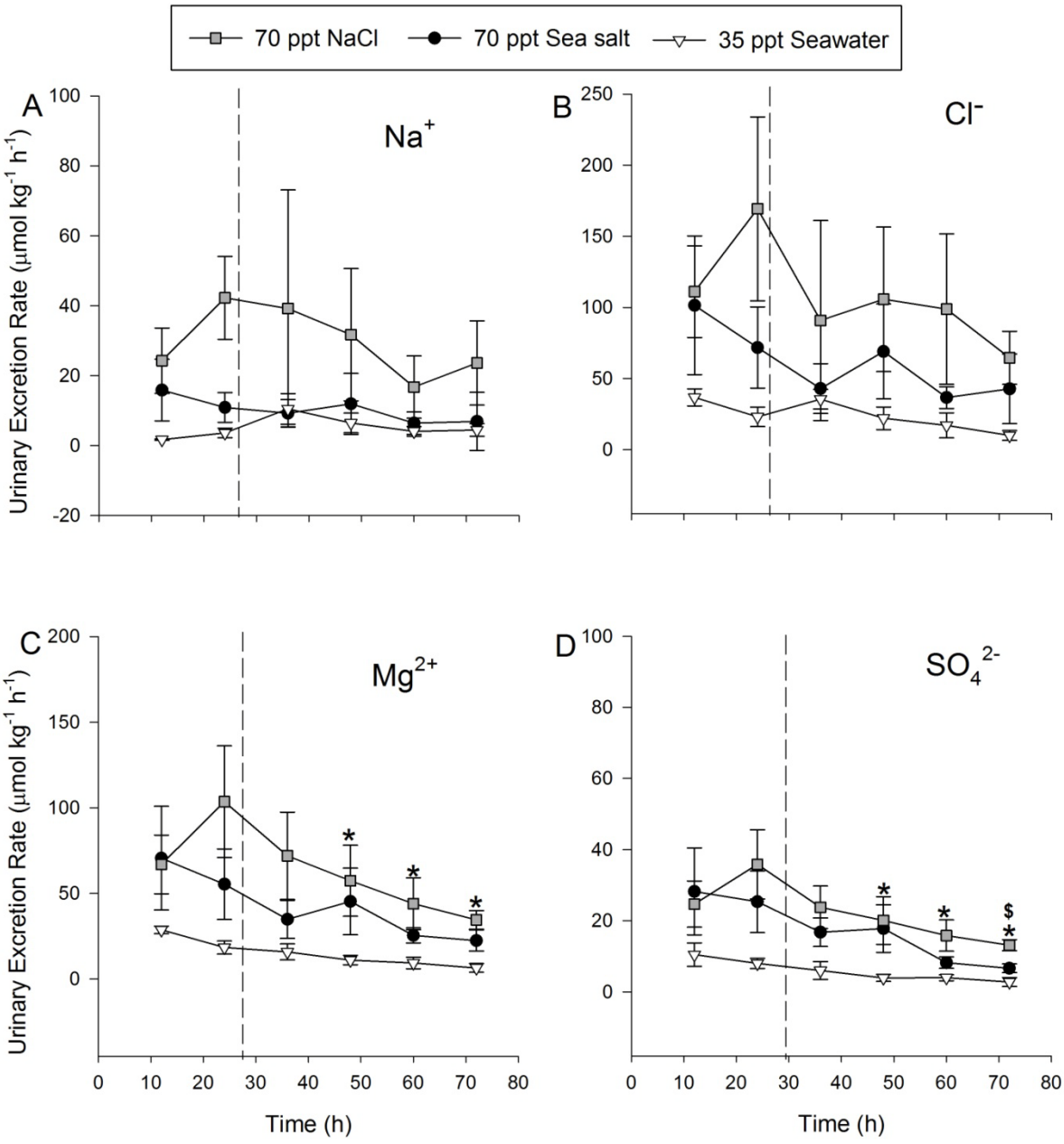


Table 3.9. Urine pH of gulf toadfish fitted with ureteral catheters exposed to 70 ppt water adjusted from 35 ppt seawater with sea salt, or adjusted with NaCl only. Values at 12 and 24 h are control observations while values from 36-72 hours were measured following transfer to hypersalinity.

Time (h)	70 ppt Sea salt	70 ppt NaCl	35 ppt Seawater
12	7.7±0.08	7.6±0.13	7.4±0.07
24	7.7±0.08	7.8±0.12	7.7±0.09
36	7.7±0.08	7.7±0.18	7.7±0.12
48	7.9±0.13	7.8±0.16	7.7±0.06
60	7.8±0.17	7.7±0.20	7.8±0.05
72	7.9±0.17	7.9±0.16	7.8±0.06

Table 3.10. Concentration of ions (mM) in urine from gulf toadfish fitted with ureteral catheters exposed to 70 ppt water adjusted from 35 ppt seawater with sea salt, or adjusted with NaCl only. Values at 12 and 24 h are control observations while values from 36-72 hours were measured following transfer to hypersalinity (n=3-8). Mg^{2+} excretion is significantly higher in both hypersaline treatments when compared to 35 ppt seawater using two-way ANOVA, but individual means were not different from corresponding control values.

	70 ppt Sea salt				70 ppt NaCl				35 ppt Seawater			
	Na^+	Cl^-	Mg^{2+}	SO_4^{2-}	Na^+	Cl^-	Mg^{2+}	SO_4^{2-}	Na^+	Cl^-	Mg^{2+}	SO_4^{2-}
12 h	28.9±7.43	155.8±15.20	129.7±7.44	60.6±6.36	29.4±5.63	162.2±10.68	109.4±8.82	43.4±5.40	8.0±0.64	184.3±28.14	135.2±4.88	54.3±12.45
24 h	39.1±13.39	179.1±18.79	138.3±18.38	73.8±8.81	61.4±18.57	217.1±30.02	141.2±14.48	54.0±5.24	23.0±7.36	164.0±27.31	125.0±11.50	51.4±7.96
36 h	36.1±11.77	163.8±23.71	133.9±12.37	70.4±7.33	52.6±18.70	151.9±28.81	122.6±13.56	51.7±12.01	67.2±25.80	210.9±8.24	89.9±22.08	33.7±10.52
48 h	28.8±13.28	209.0±39.55	149.3±19.97	71.9±10.53	61.3±20.35	212.9±53.31	150.3±16.37	63.4±11.88	49.3±17.94	177.6±20.16	104.0±17.03	43.6±12.61
60 h	26.1±9.24	198.2±20.82	136.0±9.57	48.4±4.85	42.9±14.21	159.6±21.49	124.2±19.69	55.9±14.58	54.2±14.85	163.5±19.86	99.4±16.49	51.6±16.24
72 h	40.2±17.46	227.5±39.87	138.4±8.93	44.1±4.91	60.4±24.96	171.5±34.97	125.9±21.69	65.0±15.33	52.5±19.70	151.2±22.32	90.2±18.76	42.3±15.75

Conclusions

In vivo Exposures

The objective of this study was to investigate how ambient [$MgSO_4$] and [NaCl] limit tolerance to acute hypersalinity exposure. Osmolalities of plasma and intestinal fluid were tightly correlated (Fig. 3.2) and increased with salinity, regardless of the composition of ambient seawater (Fig. 3.1), indicating that hypersaline exposure has a measurable effect on osmoregulation. Lower plasma and intestinal fluid osmolalities in fish exposed to NaCl-supplemented seawater (low [$MgSO_4$], high [NaCl]) than fish exposed to seawater supplemented with sea salt (high [$MgSO_4$], low [NaCl]) indicated that the former osmoregulates better than the latter (Fig. 3.1). In addition, decreased mortality at 85 ppt water in low ambient [$MgSO_4$] suggests that these fish had a better ability overall to deal with acute hypersaline exposure, despite the elevation in ambient NaCl concentrations (data not shown).

Three organs are of importance to water and salt exchange between a fish and its external environment: the gill, intestine, and kidney. Given that fish in water containing unusually high [NaCl] exhibited the best overall tolerance to hypersalinity despite a greater gradient against which the gill was required to excrete NaCl eliminates the gill as a limiting iono- or osmoregulatory factor for hypersalinity tolerance. This observation leaves the intestinal and renal processes as potential limitations on handling high ambient salinity.

Intestinal Transport Processes

As part of this study, we designed 4 sets of intestinal sac preparation experiments to consider the potential impact of luminal ionic composition, particularly with regard to $MgSO_4$ and NaCl, and also to manipulate the osmolality differentials across the tissue. Fish survived exposure *in vivo* to 70 ppt water, regardless of ionic composition, but intestinal epithelial preparations under the same conditions lost, rather than absorbed, water (Fig. 3.3A). Thus, it appears that isolated intestinal preparations do not perform as well as the intestine *in vivo* with regards to water and salt transport despite being viable and showing stable transport characteristics for > 6 hours (Grosell et al., 2005; Grosell and Genz, 2006). A discrepancy between tissue performance *in situ* and *in vitro* is common and it is important to recognize that aspects of epithelial transport that can be examined using isolated preparations would be impossible to study *in vivo*.

A potential alternative explanation for the discrepancy between *in vivo* and *in vitro* observations is that the anterior intestine which was used for the *in vitro* studies is not fully representative of the entire intestinal tract. Indeed, fluids in the posterior intestine exhibit the highest $MgSO_4$ concentrations and often the lowest NaCl concentrations. However, the majority of NaCl and water absorption by the intestine occurs in the anterior region and luminal fluid composition differences along the intestinal tract are relatively minor (Grosell, 2006). Nevertheless, differences in the function of the intestinal segments with regards to iono- and osmoregulation offer an exciting area for further research.

Water and ion fluxes across intestinal sac preparations under conditions mimicking intestinal fluid chemistry observed *in vivo* demonstrated that water absorption

was hindered under conditions relevant for 70 ppt seawater supplemented with sea salt, thus a normal $MgSO_4$:NaCl ratio (Fig. 3.3A). Furthermore, these experiments revealed much less impact on intestinal fluid chemistry observed in fish exposed to the same ambient salinity but with a lower $MgSO_4$:NaCl ratio, *i.e.* 70 ppt seawater supplemented with NaCl alone. These observations are in agreement with the *in vivo* observations of lower tolerance to hypersalinity in waters with normal $MgSO_4$:NaCl ratios. The differences in water and ion fluxes observed between preparations exposed to the two different *in vivo*-like conditions can be attributed to the difference in both the ionic composition of the luminal fluid and the osmotic pressure. Addition of mannitol to the luminal saline that mimicked the intestinal fluid composition of fish in water supplemented with NaCl (low $MgSO_4$:NaCl) raised the osmolality to that of the saline that mimicked intestinal fluid from fish residing in seawater supplemented with sea salt (normal $MgSO_4$:NaCl) but reduced the rate of apparent fluid secretion to a lesser extent than the saline which varied in both osmolality and $MgSO_4$:NaCl (Fig. 3.3A). An obvious interpretation of these observations is that increasing the luminal NaCl concentration provided more substrate for solute-coupled water absorption. In addition, decreased luminal [$MgSO_4$:NaCl] results in lower concentrations of $MgSO_4$ and thus less impact on luminal osmolality influencing water transport.

Increased luminal osmolality decreased water absorption regardless of luminal ionic composition (Fig. 3.5A); however, the greatest water flux occurred in the high [NaCl] treatment. High luminal [NaCl] allows for greater uptake of NaCl, and for water uptake against a greater osmotic gradient. Under *in vivo*-like conditions, the isolated toadfish intestine is capable of absorbing water against an osmotic gradient of up to ~35

mosm kg^{-1} [the difference between serosal osmolality (315 mosm kg^{-1}) and the mucosal osmolality at which water can no longer be absorbed (350 mosm kg^{-1}), see Fig. 3.5A]. The ability of the toadfish intestine to absorb water against an osmotic gradient is almost doubled (~65 mosm kg^{-1}) by increasing luminal NaCl from 90 to 150 mM and reducing $MgSO_4$ from 90 mM to 1 mM. This agrees with previous work with perfused eel intestine demonstrating that the osmotic pressure against which water absorption could be maintained was correlated with NaCl transport rates, and that both NaCl absorption and the osmotic pressure resulting in zero water flux were greater in fish acclimated to higher salinity (Skadhauge, 1974).

In vivo, precipitation of $CaCO_3$ occurs in the intestinal lumen due to alkaline conditions, high total CO_2 levels, and ingestion of Ca^{2+}-rich seawater (Wilson and Grosell, 2003; Grosell, 2006). This precipitation has been estimated to result in a reduction of luminal osmotic pressure of up to 70 mosm kg^{-1} (Wilson et al., 2002). In the context of the present findings, $CaCO_3$ precipitation would appear to be very important for water absorption. Under *in vivo*-like conditions, fluid absorption became limited against gradients of 35 mosm kg^{-1} (Fig. 3.5A); hence in the absence of a reduction in luminal osmotic pressure resulting from $CaCO_3$ precipitation (70 mosm kg^{-1} (Wilson et al., 2002)) intestinal fluid absorption might be compromised, and thus so would be survival in seawater.

Water absorption by intestinal epithelia was eliminated by the addition of 60 mM $MgSO_4$ (Fig. 3.6A). This result indicates that water absorption is greatly limited by the increased luminal [$MgSO_4$] and resulting high osmolality. There was little change in luminal $MgSO_4$ concentrations over the length of these experiments, accounted for by the

limited water absorption and $MgSO_4$ secretion. NaCl absorption persisted despite elevated luminal $MgSO_4$ (Fig. 3.6B), demonstrating that the driving force for solute-coupled water absorption was maintained under these conditions and luminal to serosal water movement was likely unaltered by luminal $MgSO_4$. Therefore, the observed reduction in net water absorption can be viewed as a product of back-flux of water from the serosal to mucosal fluids, driven by the osmotic gradient created by $MgSO_4$. Under conditions where osmolality was elevated using mannitol instead of $MgSO_4$ (Fig. 3.5), water absorption was similarly reduced. Increased absorption of Cl^- when luminal osmolality was increased (Fig. 3.5B) coincided with decreasing water absorption, further supporting the idea that reduction of water uptake was due to high luminal osmotic pressure, whether produced by mannitol or $MgSO_4$.

Absorption of Na^+ and Cl^- by the intestinal epithelium primarily occurs via Na^+-K^+-$2Cl^-$ cotransport (NKCC) and Na^+-Cl^- cotransport (NCC) (Marshall and Grosell, 2005), with Cl^- absorption also taking place in exchange for HCO_3^- secretion (Wilson et al., 2002; Grosell et al., 2005; Grosell, 2006). Overall the present study reveals increased HCO_3^- secretion under lower luminal Cl^- concentrations and increased osmolality (Fig. 3.5E). Conversely, when luminal ions are dominated by NaCl, significantly greater Cl^- absorption in conjunction with decreased HCO_3^- secretion is observed. Observations of reduced HCO_3^- secretion yet increased Cl^- absorption with increasing luminal NaCl concentrations indicates a decreased importance of Cl^-/HCO_3^- exchange at high [NaCl]. These observations are in agreement with earlier reports of elevated Cl^-/HCO_3^- exchange when luminal NaCl concentrations are low and conditions for NaCl co-transport become less favorable (Grosell and Taylor, 2007). In addition to increased Na^+ and Cl^- absorption

in high [NaCl], greater net K^+ secretion across the tissue at high luminal [NaCl] was also observed (Fig. 3.4D). Plasma [K^+] and [Ca^{2+}] are lower when luminal fluid has high [NaCl] and low [$MgSO_4$], particularly at 70 ppt salinity and higher (Table 3.7). The effect on K^+ transport is likely associated with K^+ entering the lumen via an apical K^+ channel (Musch et al., 1982), to be recycled via uptake by NKCC, allowing for enhanced uptake of Na^+ and Cl^-.

Under *in vivo*-like conditions, when Cl^- is being absorbed by both NKCC and Cl^-/HCO_3^- exchange, Cl^- absorption exceeds Na^+ absorption. However, high luminal [NaCl] in hypersalinity diminishes the difference between Cl^- and Na^+ absorption rates (Fig. 3.4B). The decrease in HCO_3^- secretion and increase in absorption of Cl^- and Na^+, while the difference in flux between Cl^- and Na^+ absorption decreases, suggests that when luminal NaCl is increased there is a shift from Cl^- absorption via both NKCC and Cl^-/HCO_3^- exchange to Cl^- absorption primarily via the NKCC and NCC cotransport systems. Luminal osmolality does not affect absorption of Na^+, but does increase Cl^- absorption and HCO_3^- secretion (Fig. 3.5B, E), suggesting an increased role for Cl^-/HCO_3^- exchange rather than co-transport pathways when intestinal fluid osmotic pressure is increased.

All treatment groups display an apparent intestinal secretion of $MgSO_4$ with a trend towards greater $MgSO_4$ secretion when exposed to approximately equal luminal [NaCl] and [$MgSO_4$] (Fig. 3.5C). The limited uptake of Mg^{2+} and SO_4^{2-} despite substantial inward directed gradients in many of the present experiments suggests low permeability of the intestinal epithelium to these ions. At least for SO_4^{2-} this apparent impermeability is due to secretion of SO_4^{2-} in exchange for Cl^- uptake which acts to mask

diffusive gain of this divalent anion (Pelis and Renfro, 2003). Whether intestinal secretion of Mg^{2+} is occurring similarly is currently unknown but offers an exciting area for further studies. Interestingly, secretion of Mg^{2+} and SO_4^{2-} was observed in the present study largely from preparations showing the lowest water absorption rates whereas preparations with high water absorption rates showed negligible secretion of these divalent ions. A possible explanation for this observation is that high water absorption rates contribute to Mg^{2+} and SO_4^{2-} absorption by solvent drag, an absorption which is exceeded by the tissues' capacity for secretion when water absorption rates are low.

Overall, it appears that intestinal water absorption, which is impaired by elevated luminal $MgSO_4$ and reduced NaCl, may present a limitation for osmoregulation at extremely high salinities. However, the possibility that some $MgSO_4$ is absorbed across the intestinal epithelium *in vivo*, increasing the demand for renal excretion and associated water loss and that this process also may limit survival in extreme saline waters, cannot be dismissed.

Renal Contribution

We note that the urine collected in this study indicates only the effects of ion and fluid transport by the kidney, as the urinary bladders were tied off and their secretory and absorptive functions therefore precluded from impacting urine composition. Comparisons of bladder urine and ureteral urine in *O. beta* indicate significantly higher concentrations of monovalent ions in the latter, suggesting reabsorption of Na^+ and Cl^- by the urinary bladder (McDonald et al., 2000). However, urea concentrations are not

significantly different between bladder and ureteral urine *in vivo* (McDonald et al., 2000) and net water flux rates using an *in vitro* urinary bladder preparation are not significantly different than zero (McDonald et al., 2002b) suggesting that fluid absorption by the toadfish urinary bladder is likely not substantial in control, seawater-acclimated fish. This is in contrast to the oyster toadfish (*Opsanus tau*), in which 60% of urine excreted by the kidney is believed to be absorbed by the bladder, primarily driven by Na^+ and Cl^- uptake (Howe and Gutknecht, 1978). The function of the urinary bladder in *O. beta* appears to be more similar to another close relative, the plainfin midshipman (*Porichthys notatus*) in which urea, Na^+, and Cl^- concentrations in the bladder and ureteral urine are not significantly different from each other, supporting a minimal water reabsorptive capacity of the bladder in this fish under control seawater conditions (McDonald et al., 2002a). However, in a separate study by McDonald and Grosell (2006), *O. beta* acclimated to high salinities (50 and 70 ppt seawater made up with sea salt) had significantly lower bladder urine Na^+ and SO_4^{2-} concentrations and, at the highest salinity, significantly higher osmolality and concentrations of Cl^-, Mg^{2+}, and urea, compared to fish acclimated to control, seawater conditions (33-35 ppt), suggesting an overall increase in water absorptive capabilities of the urinary bladder in fish acclimated to high salinity. In comparison, toadfish acclimated to the same conditions in the present study (70 ppt sea salt) showed little change in ureteral urine composition (Table 3.10), suggesting that at high salinities the urinary bladder of *O. beta* may play a more substantial role in water balance than the kidney.

In agreement with previous studies (Chapter 2), hypersalinity had a very minimal impact on plasma Mg^{2+} levels (Table 3.7), suggesting that limitations in renal clearance

of this ion were not contributing to the difference in tolerance seen between fish exposed to seawater supplemented with NaCl (low $MgSO_4$:NaCl) and fish exposed to hypersalinity with normal ionic ratios (seawater supplemented with sea salt). Surprisingly, urine production was elevated in both hypersaline treatments (Fig. 3.7A), but as mentioned above, the role of the urinary bladder in urine formation and water retention likely increases with increased salinity; uncatheterized toadfish acclimated to 70 ppt seawater have an osmotic gradient of 47 mOsm between plasma and bladder urine, compared to 24 mOsm in 33 ppt seawater (McDonald and Grosell, 2006). Therefore, the increase in urine flow rate measured in both groups of hypersaline fish may not have been evident in fish with a fully intact renal system, *i.e.*, if the urinary bladders were not bypassed as in the current study. Regardless, the reduced survival of toadfish exposed to elevated ambient salt concentrations containing normal seawater $MgSO_4$:NaCl ratios appears not to be related to increased renal water loss associated with renal tubule processes, such as an increase in tubular secretion of Mg^{2+} and SO_4^{2-}. Indeed, toadfish exposed to 70 ppt seawater supplemented with NaCl alone displayed greater urine excretion rates and thus greater water loss, likely due to an increase in NaCl secretion by the proximal tubule and/or a reduction in renal NaCl reabsorption by the collecting duct as a way to maintain plasma osmolality. However, it should be kept in mind that the role of the urinary bladder in these two hypersaline groups was not tested and the potential impairment in its function at these high salinities as a contributing factor to the reduction in tolerance in fish exposed to elevated salinity containing normal seawater $MgSO_4$:NaCl ratios has not been ruled out in its entirety.

The renal excretion of Mg^{2+} and SO_4^{2-} was elevated at the later time points in the fish exposed to hypersaline waters supplemented with NaCl (low $MgSO_4$:NaCl), compared to 35 ppt seawater (Fig. 3.8C, D), supporting the finding that fish exposed to hypersaline waters supplemented with NaCl had the highest intestinal $MgSO_4$ uptake and therefore the greatest need for renal clearance of these ions. As discussed above, $MgSO_4$ absorption across the intestine may be elevated due to solvent drag, as the intestine of fish exposed to 70 ppt water supplemented with NaCl alone is better able to absorb water. Flux of Mg^{2+} and SO_4^{2-} across isolated intestinal epithelia of fish exposed to luminal saline mimicking 70 ppt water supplemented with sea salt was not significantly different from luminal saline mimicking 70 ppt water supplemented with NaCl alone. However, this may be due to differences between *in situ* and *in vitro* methods, as discussed above. Another possible explanation for this difference may be the function of the anterior intestine with regards to ion transport, which may not be representative of the response of the intestine as a whole. Further, greater urinary excretion of Mg^{2+} and SO_4^{2-} in the fish exposed to hypersaline water supplemented with NaCl may be accounted for by the increased ureteral urine flow rate of these fish. Higher rates of urinary $MgSO_4$ excretion as a result of increased water loss are likely due to a reduction in renal NaCl reabsorption and/or an increase in NaCl secretion by the proximal tubule to maintain constant plasma osmolality.

Perspectives and Significance

In conclusion, the present study demonstrates that branchial and renal functions do not limit survival of teleosts exposed to extremely hypersaline waters. Rather, intestinal

water absorption appears to be the limiting physiological process for tolerance to high ambient salinity. While increased luminal osmotic pressure at high salinity does contribute to the prevention of water absorption, we have demonstrated that the ionic composition of the luminal fluid, particularly [$MgSO_4$] and [NaCl], dictates the epithelial capacity for net water absorption. Overall, we conclude that concentrated $MgSO_4$ within the intestinal lumen impairs water absorption and limits survival of toadfish in extreme hypersaline environments.

A variety of natural conditions exposes fish to hypersalinity, from small, transient tidal pools and salt marshes, to anthropogenic salt concentrating ponds and other inland waters (Herbst, 2001; Molony and Parry, 2006), to long-term concentration of lakes or entire estuarine basins (Pagès and Citeau, 1990; Harrison, 2004), emphasizing the need to understand the physiological constraints on vertebrate life in aquatic environments of increasing salinity. While study of fish exposed to hypersalinity in a laboratory setting is useful for understanding the physiology of osmoregulatory mechanisms, hypersalinity is clearly a challenge often experienced within the natural environment, and is therefore also interesting from an ecological viewpoint. Furthermore, many hypersaline environments, particularly inland salt lakes, differ in ionic composition from marine environments (Herbst, 2001). Teleost fish represent approximately half of all vertebrate species and it is interesting to consider how this group has developed the physiological abilities to inhabit such a large range of environments that challenge maintenance of internal ion and water balance. Understanding of these regulatory processes in teleost fish may provide a baseline for research in other vertebrates, including mammals.

Chapter 4

Intestinal transport following transfer to increased salinity in an anadromous fish (*Oncorhynchus mykiss*)

Summary

For a small group of fish species, including the anadromous rainbow trout (*Oncorhynchus mykiss*), the ability to transition from a freshwater to seawater environment is an intrinsic requirement of the species' life history. The dramatic differences between hyper- and hypoosmoregulation are developed quickly, on the order of hours to days, and at all scales, from gene expression to organ function. This study expands our understanding of intestinal ion and water transport in *O. mykiss* following acute transfer to 70% seawater (SW), by examining isolated tissue preparations and mRNA expression of ion transporters important to both intestinal osmoregulation and maintenance of acid-base balance. Plasma [Mg^{2+}] was increased from FW values at 24 h but was recovered by 72 h. At the same time, total CO_2 in the intestinal fluids was found to increase with SW exposure/acclimation and luminal [Na^+] decreased after 24 h of SW exposure. Water absorption occurred in all intestinal preparations following transfer from FW to SW. Overall, *in vitro* experiments demonstrated the importance of base secretion to epithelial water uptake, and suggested the primary physiological adjustments occurred 24-72 h after acute SW transfer. A Na^+/H^+ exchanger (NHE2) and Cl^-/HCO_3^- exchanger (SLC26a6) were hypothesized to be important intestinal transporters of acid-base equivalents, Na^+, and Cl^-. These transporters were not uniformly expressed across tissue samples, and expression, where present, did not change following salinity transfer.

NHE1, however, was expressed in all examined tissues (gill, kidney, anterior intestine, and pyloric cecae), and the pyloric cecae showed increased expression following salinity transfer.

Background

Marine teleost fish regulating body fluids at a lower osmotic pressure than that of the surrounding medium constantly lose water to their surroundings, and compensate by drinking. The ingested fluid is modified by the gastrointestinal tract, with absorption of ions, particularly Na^+ and Cl^-, driving water uptake (Skadhauge, 1974; Usher et al., 1991). The absorbed Na^+ and Cl^- are subsequently excreted at the gill allowing for water gain which offsets the diffusive water loss (Marshall and Grosell, 2005; Evans et al., 2005). Conversely, fish in freshwater are faced with water influx and ion efflux to their surroundings, and compensate for these passive fluxes by producing dilute urine and actively taking up ions at the branchial epithelium (Perry et al., 2003b). Many fish, however, migrate between these two environments, which often are not solely marine or freshwater, but of an intermediate salinity. Thus, the ability of fishes to transition between the two distinct regulatory systems in either seawater or freshwater is an essential trait. Understanding the physiological characteristics of this transition will allow for a better understanding of each regulatory system and their most essential components for water and ion transport.

Upon transfer to seawater, rainbow trout (*Oncorhynchus mykiss*) experience high mortality (Landless, 1976; Johnston and Cheverie, 1985). However, the stress of salinity transfer is reduced by gradual acclimation or greater size (Johnston and Cheverie, 1985;

Fuentes et al., 1997). Alternatively, rainbow trout of all sizes are able to tolerate transfer to salinities lower than regular seawater, up to 28 ppt (Landless, 1976; Eddy and Bath, 1979). Acclimation of trout to seawater is characterized by an initial "crisis" period (8-30 h) during which the principal increases in ion influx and other physiological changes occur in response to the onset of drinking. This is followed by a longer stabilization period where more permanent regulatory changes take place, including increased activity of intestinal and branchial Na^+-K^+-ATPase (Colin et al., 1985; Johnston and Cheverie, 1985; Grosell et al., 2007). Trout are considered fully acclimated at 7-10 days post-transfer (Bath and Eddy, 1979). The steelhead strain of rainbow trout used in this study are capable of acclimation to seawater as adults, and the smolts, although freshwater inhabitants, are able to be acutely transferred to 70% seawater and maintained indefinitely at this salinity with little mortality.

Recent studies have examined the transport mechanisms essential to intestinal osmoregulation in rainbow trout exposed to increased salinity. In particular, a cytosolic isoform of carbonic anhydrase (CA) has been shown to account for the majority of cellular production of HCO_3^- secreted into the intestinal lumen in exchange for Cl^- (Grosell et al., 2009a). The acid-base equivalents resulting from CA-catalyzed CO_2 hydration in the trout (Grosell et al., 2009a) are transported across the cellular membrane by the Na^+-H^+ exchanger 3 (NHE3) and apical V-type H^+ ATPase, both of which are upregulated following transfer to increased salinity (Grosell et al., 2007; Grosell et al., 2009a) and HCO_3^- across the apical membrane, presumably by the anion exchanger SLC26a6 (PAT-1, see Mount and Romero, 2004), which has been shown to be responsible for Cl^-/HCO_3^- exchange in other teleost species (Kurita et al., 2008; Grosell

et al., 2009b). In addition, the Na^+:HCO_3^- cotransporter (NBC1) possibly plays a role in HCO_3^- import across the basolateral membrane in the trout, as has been demonstrated for other teleosts (Taylor et al., 2010). Initial investigations into the integrative role of these proteins focused on the gill and kidney in FW trout (Perry et al., 2003a), but later studies have demonstrated the importance of this system for the transport of acid-base equivalents in the intestine (Grosell et al., 2009a), and specifically during seawater acclimation (Grosell et al., 2007).

In the present study, we examined the physiological response of the freshwater (FW) acclimated rainbow trout intestine for 24 and 72 h following acute transfer to 70% seawater (SW). A combination of isolated intestinal sac and Ussing chamber pH-stat preparations were used to examine the response of rainbow trout intestine with respect to the transport mechanisms related to intestinal HCO_3^- secretion and ion movement. In addition, we investigated the transcriptional regulation of select ion transporters in response to SW transfer in the gill, intestinal epithelium, pyloric cecae, and kidney of rainbow trout. Specifically, three transporters [SLC26a6, Na^+-H^+ exchanger 1 (NHE1), and Na^+-H^+ exchanger 2 (NHE2)] were hypothesized to be important to intestinal ion transport following SW transfer. The SLC26a6 Cl^-/ HCO_3^- exchanger has been suggested as a critical ion exchanger accounting for a substantial amount of Cl^- uptake and HCO_3^- secretion in the intestine of both species in which it has been pursued, *Opsanus beta* (Grosell et al., 2009b) and *Takifugu obscurus* (Kurita et al., 2008). Furthermore, the increased HCO_3^- secretion that occurs following transfer to high salinity (Chapter 2) suggests this transporter may be of even greater importance in fish with elevated osmoregulatory demand. Additionally, previous observations in trout and other

teleost species have demonstrated renal expression of SLC26a6, most likely involved with SO_4^{-2} transport, which is dramatically increased in seawater-acclimated fish (Kato et al., 2009). These observations led us to predict that in addition to intestinal expression, SLC26a6 would also be expressed in the trout kidney and upregulated following SW transfer. Similarly, based on previous studies demonstrating that NHE2 was the predominant NHE isoform in the trout gill (Ivanis et al., 2008), we predicted branchial expression of this transporter, and increased expression in SW-exposed fish. In the intestine, transport of H^+ may occur across the apical and/or basolateral membranes. Na^+ is actively taken up at the apical membrane primarily by NKA and NKCC, and H^+ secretion in the trout has been suggested to occur via an apical V-type H^+-ATPase but Na^+/H^+ antiport is an alternative mechanism which may also be involved (Wilson et al., 1996; Grosell et al., 2007). A role for basolateral NHE in the intestine is also suggested, as H^+ extrusion is dependent on serosal Na^+ in *O. beta* (Grosell and Genz, 2006). We therefore investigated the possible expression of NHE1 (basolateral) and NHE2 (apical) in the anterior intestine and pyloric cecae. NHE1 is essential for regulation of intracellular pH in vertebrate intestine via Na^+/H^+ exchange (Noel and Pouyssegur, 1995). Furthermore, both NHE1 and NHE2 were expected to be upregulated following SW transfer to accommodate the increased requirement of the intestinal epithelium for Na^+ absorption (driving water uptake) and H^+ transport (maintaining intracellular acid-base balance) in increased salinity.

Materials and Methods

Experimental animals and salinity transfer

Steelhead rainbow trout smolts (*Oncorhynchus mykiss*) of approximately 50-70 g were obtained in July 2007 from the Robertson Creek Hatchery (Fisheries and Oceans Canada, Port Alberni, BC), and transported to Bamfield Marine Sciences Centre (Bamfield, BC). Trout were kept in13ºC, outdoor, aerated flow-through tanks containing freshwater (FW) or in 70% full-strength seawater (SW, 23-25 ppt) following gradual acclimation over two weeks, and were held at the acclimation salinity for at least 5 days prior to experimentation. Trout were also acutely transferred from FW to SW and sampled at 24 and 72 h following transfer. Fish were fed twice daily, but food was withheld for 72 h prior to experimentation.

Fluid sampling

Plasma samples were obtained from anaesthetized fish by caudal puncture. Briefly, a 21-gauge needle fitted to a 1 ml syringe (BD Syringe, Franklin Lakes, NJ, US) rinsed with heparinized mucosal saline (see Table 4.1) was inserted ventrally into either the caudal vein or artery and ~200 µl of blood was withdrawn. The blood was immediately centrifuged to separate the plasma, which was analyzed for total CO_2 (tCO_2) and then stored at -20˚C for further analysis (see below). Following blood sampling, fish were sacrificed by a cephalic blow. Intestinal fluids were sampled by clamping the intestine proximal to the pyloric cecae and proximal to the rectal sphincter, removing the entire segment, and gently massaging the tissue to drain the fluid into a tube. Fluids were

analyzed for tCO_2 and then stored at -20˚C for further analysis. Intestinal fluids collected from FW trout could not be analyzed due to low volumes.

Intestinal sac preparations

Intestinal sac preparations were used to measure water and ion fluxes across the intestinal epithelium and were made according to the following protocol, modified from Grosell and Taylor (2007). Fish were sacrificed by a blow to the head followed by spinal transection, and the anterior intestinal segment was isolated. A filling catheter of PE 50 tubing (I.D. 0.58 mm, O.D. 0.965 mm), heat flared on one end, was inserted into the anterior end of the intestinal segment and tied in place with 3-0 silk sutures. The lumen was rinsed with 5 ml of mucosal saline (Table 4.1) to remove any intestinal contents and the distal end of the tissue segment was tied closed with 3-0 silk. The resulting sac preparation was filled with mucosal saline, sealed, and the initial full weight of the sac was determined. The 1 ml Hamilton syringe containing the filling saline was weighed before and after filling the sac preparation to determine the exact volume of saline added to the sac. To ensure that the remaining saline in the syringe was reflective of the saline contained in the sac preparation, saline was flushed back and forth between the sac preparation and the syringe three times prior to sealing the filling catheter and the remaining saline was kept as the initial luminal saline sample. The filled sac was placed in a scintillation vial containing 15 ml serosal saline (Table 4.1), which was gassed with a 0.3% CO_2/O_2 mix for at least 30 minutes before and throughout the 2 h flux period. At the end of the flux period, the sac preparation was removed from the vial, blotted dry, and weighed to determine the final full sac weight. The sac was cut open at the distal sutures

and the luminal saline was collected in a tube. Subsequently, the sac was cut open longitudinally and blotted dry, and the weight of the empty sac including the filling catheter and sutures was determined. The catheter and sutures were removed and the tissue segment was stored overnight at 4˚C to allow the tissue to relax. Surface area was determined by tracing the spread-out tissue segment on paper, weighing the traced area, and correcting against the weight of 1 cm^2 paper. Initial and final luminal saline samples were measured for pH, tCO_2, osmotic pressure, and ionic composition as described below.

pH-stat titrations

Electrophysiological recordings as well as measurements of luminal net base secretion by pH-stat titration in Ussing chambers were performed on isolated intestinal epithelia as described in detail previously (Grosell et al., 2009a). The anterior segment of the intestine (~1 cm tissue immediately distal to the pyloric cecae) was cut longitudinally, mounted in a tissue holder (Physiological instruments, P2404), exposing 0.25 cm^2 gross surface area, and placed between Ussing-style half chambers (Physiological instruments, 2413), each containing 1.6 ml of appropriate saline (Table 4.1) and maintained at 13±1ºC throughout experimentation. Saline in the serosal half-chamber was mixed with humidified 0.3% CO_2 in O_2 gas, and the luminal saline with O_2 for at least 30 min before, and throughout the titration experiments.

Transepithelial potential (TEP) was measured under asymmetrical, *in vivo*-like conditions. Current and voltage electrodes (Physiological Instruments P2020-S) connected to amplifiers (Physiological instruments VCC600) passed 3 s pulses of 30

μAmp from the mucosal to the serosal side every 60 s under current clamp conditions and the resulting TEP measurements were logged on a personal computer using a data acquisition system (BIOPAC systems interface with AcqKnowledge software, v3.8.1). The change in TEP due to the current pulses was used to calculate the conductance (μSi) of the epithelium. For determination of net base secretion by the anterior intestinal epithelium, a pH combination electrode (Radiometer PHC4000-8) and a micro-burette tip were placed in the luminal half chamber, both of which were connected to a pH-stat titration system (Radiometer, TIM 854 or 856). Base secretion was determined from the rate of addition of 0.005 N HCl to maintain luminal pH at 7.800 ± 0.003. Luminal pH and rate of acid addition were logged on personal computers using Titramaster software (v1.3 and 2.1, Radiometer). Data were collected for 60 min following stabilization of base secretion, TEP, and conductance. Preparations were run side-by-side on two parallel and identical set-ups, and fish from each experimental group were run on alternating set-ups to avoid potential experimental set-up biases.

Analysis of fluid samples

Intestinal fluids, plasma, and saline from the experiments described above were all analyzed using the same methods. Blood plasma and intestinal fluids were analyzed for tCO_2 using a total CO_2 analyzer (Corning 965, Medfield, MA, US). Concentrations of SO_4^{2-} and Cl^- were quantified by anion chromatography (Dionex 120, Sunnyvale, CA, US), while Na^+, Mg^{2+}, K^+, and Ca^{2+} were analyzed by fast sequential flame atomic absorption spectrometry (Varian 220, Palo Alto, CA, US) using an air/acetylene flame. Ion flux across intestinal sac preparations was calculated as the difference between the

initial and final amounts of ion (μmol) in the lumen per cm^2 tissue per hour, while water flux was calculated as the difference in mass of the filled preparation before and after the flux period per cm^2 tissue per hour.

Analysis of mRNA expression following salinity transfer

Trout were sacrificed by cephalic blow from freshwater and after 24 h, 72 h and >12 days in 70% seawater and pyloric cecae, anterior intestine, gill, and kidney tissue were collected, immediately frozen in liquid nitrogen, and stored at -80˚C. Tissue samples were collected from 8 fish at each time point. Anterior intestinal tissue was often used for the physiological experiments outlined above, rather than for expression analysis, resulting in a low sample size for the FW, 24 h, and 72 h time points. The mRNA was isolated by homogenization of ~50 mg tissue in 1 ml RNA STAT-60 (Tel-Test Inc.), extracted as per the manufacturer's instructions, and resuspended in 20 μl nuclease-free water. Isolated mRNA (10 μg, calculated from optical density) was then treated with TURBO DNA-*free* Kit (Applied Biosystems) according to kit instructions. A SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen) was used for cDNA synthesis, following the manufacturer-provided protocol, using random hexamer primers. Subsequent cDNA was diluted 1:10 in molecular biology grade water, and stored at -20˚C. Expression of mRNA was assessed by real time RT-PCR using a Brilliant SYBR Green QPCR Master Mix kit (Stratagene) and MX3005P instrument (Stratagene). Reactions (12.5 μl final volume) were prepared using 1 μl cDNA template, 6.25 μl Master Mix, and 0.5 μl primer set (stock concentrations: 3.75 μM for NHE2, NBC1, V-ATPase, 18S; 2.5 μM for NHE1 and SLC26a6) (Table 4.2). PCR products

obtained using SLC26a6 and NHE1 primers were sequenced to confirm that the primers amplified the target genes. Reaction temperatures over 40 cycles were 58˚C annealing (1 min), and 72˚C extension (1 min). Amplification efficiencies were determined from standard curves generated by serial dilution of a pooled cDNA sample. The expression values were assessed using the ΔΔCt method (Pfaffl, 2001) against the 18S control gene (cDNA diluted 1:1000).

Disassociation curves were used to both ensure the presence of a single amplicon per reaction, and to ensure that calculated Ct values did not result from the presence of primer dimers. For all genes, primer dimers were completely absent provided the presence of the appropriate template (according to standard curve dilution series). Expression was considered non-detectable if calculated Ct values were the result of primer dimers, which was verified by the occasional presence of primer dimer based Ct values in negative controls. For some genes, not all individuals in an experimental group showed detectable expression (Table 4.3) and treatment groups where the majority of samples were considered non-detectable were excluded from analysis of relative expression levels.

Statistical analysis

All values are given as mean ± s.e.m. For pH-stat experiments, base secretion, TEP, and conductance control values were determined from the mean values of the last 30-60 minutes of the 60 min control period and compared using one-way ANOVA to analyze differences between the two acclimation groups (FW and SW), and 24 h and 72 h post-transfer time points. Gut sac fluxes were also analyzed using one-way ANOVA.

Table 4.1. Nominal saline compositions (in mmol l^{-1}) for pH-stat titration and intestinal sac preparation experiments on isolated intestinal epithelia. Measured serosal [HCO_3^-] was 5.1, pH was 7.8. Salines were adjusted to 310 mOsm kg^{-1} with NaCl (serosal) or mannitol (mucosal). Luminal saline was gassed with 0.3% CO_2/O_2 at least 30 min prior to and throughout experimentation, while serosal saline was similarly gassed with O_2.

	Serosal saline	Mucosal saline
NaCl	151	69
KCl	3	5
$MgSO_4$	0.88	77.5
$MgCl_2$	-	22.5
Na_2HPO_4	0.5	-
KH_2PO_4	0.5	-
$CaCl_2$	1	5
$NaHCO_3$	5	-
Glucose	5	-
HEPES (Free acid)	3	-
HEPES (Na salt)	3	-

Table 4.2. Primers used in qPCR reactions to assess expression of mRNA of the V-type H^+-ATPase (V-ATP), Na^+/HCO_3^- cotransporter (NBC1), Na^+/H^+ exchangers (NHE1, NHE2), and anion exchanger (SLC26a6) genes.

Gene	Forward Primer	Reverse Primer
18S[a]	5'-TCTCGATTCTGTGGGTGGT-3'	5'-CTCAATCTCGTGTGGCTGA -3'
V-ATP[a]	5'-ATGTGGACAGACAGCTGCAC -3'	5'-CATCAGCATGGTCTTTACGG -3'
NBC1[a]	5'-TGGACCTGTTCTGGGTAGCAA-3'	5'-AGCACTGGGTCTCCATCTTCAG-3'
NHE1	5'-CCAGCTCTTCTTCCTGTGCCT-3'	5'-CAGCAGGTCAACTCCGCTCA-3'
NHE2[b]	5'-TGTGCCCTGACCATGAAGTA -3'	5'-CCCAGTTCCACTCGTGTTCT -3'
SLC26a6	5'-CCTACCTGTCGGAGCCACTG-3'	5'-TTGGAGCACACCTCCACCAG-3'

[a] (Grosell et al., 2007)
[b] (Ivanis et al., 2008)

Table 4.3. Fraction of total qPCR reactions (template isolated from individual fish) that had detectable mRNA expression.

		Pyloric Cecae	Anterior Int.	Kidney	Gill
NHE1	FW	4/7	2/2	8/8	7/8
	24 h	8/8	3/3	8/8	7/8
	72 h	7/8	2/2	7/8	6/8
	SW	7/8	6/8	7/7	7/8
NHE2	FW	2/7	0/2	5/8	8/8
	24 h	--	1/3	1/8	8/8
	72 h	--	1/2	3/8	8/8
	SW	2/8	2/8	3/7	8/8
SLC26a6	FW	1/7	1/2	8/8	0/8
	24 h	--	1/3	8/8	6/8
	72 h	--	0/2	8/8	4/8
	SW	2/8	1/8	7/7	2/8
V-type	FW	7/7	2/2	--	--
	24 h	8/8	3/3	--	--
	72 h	8/8	2/2	--	--
	SW	8/8	7/8	--	--
NBC1	FW	7/7	--	--	--
	24 h	8/8	--	--	--
	72 h	8/8	--	--	--
	SW	8/8	--	--	--

Relative mRNA expression was assessed using one-way ANOVA against the FW control which was set to a relative value of 1. Individual means identified as significant by the ANOVA were then analyzed against the control (FW) using a Bonferroni t-test. Data that were not normally distributed were analyzed using Kruskal-Wallis one-way ANOVA on ranks and significant means analyzed post hoc using Dunn's method. Differences were considered significant at $P<0.05$.

Results

In vivo samples

Plasma tCO_2 did not differ significantly among groups, although there appeared to be a trend toward increasing tCO_2 over the salinity transfer time course (Table 4.4). The only change in plasma ion concentrations following salinity transfer was a significant increase from FW values in $[Mg^{2+}]$ at 24 h (Table 4.4). Intestinal fluid volumes from fish in FW were too low to be measured for ion composition. In the intestinal fluids, tCO_2 was significantly lower, and the concentration of Na^+ significantly higher, at 24 h than in fish 72 h after transfer or fully acclimated to SW (Fig. 4.1). There appeared to be increased $[Mg^{2+}]$ at 72 h and in trout fully acclimated to SW, however this was not significant when assessed by one-way ANOVA ($P<0.066$). Intestinal fluid pH (8.28±0.06) did not change following transfer to SW (data not shown).

Response of isolated tissue

Intestinal sac preparations were used to compare the physiological response of isolated intestinal tissue presented with salines mimicking extracellular and gastrointestinal fluids

of fish acclimated to SW (Table 4.1). Water absorption by intestinal sacs occurred in all treatments (Fig. 4.2A), but did not change following SW transfer. While there was no significant change in secretion of tCO_2, it did appear to increase at the intermediate timepoints (Fig. 4.2B). There was no change in flux across the intestinal epithelium of any of the measured ions (Fig. 4.3).

Using pH-stat titration in Ussing chambers, no difference was observed over the course of the 60 minute experiment in transepithelial potential (TEP) or conductance (G) of isolated anterior intestinal tissue from trout acclimated to FW, SW, or 24 and 72 h after acute SW transfer (Table 4.5). However, there was a trend toward decreased TEP at 24 h (P<0.085), which, interestingly, occurred with a slight reduction in conductance (P<0.092). Base secretion in FW and at both intermediate timepoints was significantly lower than in trout fully acclimated to SW (Fig. 4.4).

mRNA expression following SW transfer

Expression of V-type H^+ ATPase and NBC1 in the pyloric cecae increased following transfer to SW (Fig. 4.5). Although expression changes of both V-type ATPase and NBC1 in the pyloric cecae showed a significant effect of acclimation time when assessed using one-way ANOVA, individual means were not significant at any time point when compared to FW. Expression of V-type H^+ ATPase was also examined in the anterior intestine, but unlike the increased expression observed in the pyloric cecae, was not significantly different following transfer to SW (P<0.516).

In addition to these two previously studied genes, expression of NHE1, NHE2, and SLC26a6 was examined in the pyloric cecae, anterior intestine, kidney, and gill.

NHE1 was expressed in the majority of tissue samples examined, with the lowest rate of expression occurring in the FW pyloric cecae (Table 4.3). The overall trends in expression varied little when samples without detectable product (Table 4.3) were removed from the analysis (Fig. 4.8). Analysis including only tissues that expressed NHE1 showed that NHE1 expression in the anterior intestine, kidney, gill, and pyloric cecae (Fig. 4.8) did not differ significantly over the salinity transfer time course, relative to FW expression. Expression data for NHE2 were analyzed for the gill (Fig. 4.6), but NHE2 expression was not detectable in the pyloric cecae, anterior intestine, or kidney. Compared to expression values for the FW control group, expression of NHE2 mRNA in the gill did not differ over the salinity transfer time course (Fig. 4.6). Similarly, expression of SLC26a6 was analyzed for the kidney, but was not detectable in the pyloric cecae, anterior intestine, or gill. Expression of SLC26a6 mRNA in the kidney following transfer to SW did not differ from FW values (Fig. 4.7).

Conclusions

In vivo observations

Surprisingly, very few changes were observed in the ionic composition of blood plasma following transfer to SW (Table 4.4), with the only significant increase occurring in $[Mg^{2+}]$ at 24 h. These results are very similar to those observed by Fuentes *et al* (1997) where non-anadromous trout were gradually transferred to SW over 15 days. Similarly, there were no differences in plasma Na^+, Cl^-, or K^+ between fish acclimated to FW and 2/3 SW (Eddy and Bath, 1979) . It has been previously observed, however, that influx of monovalent ions increases very quickly following acute transfer of trout to seawater

Table 4.4. Concentrations (mM) of Na^+, Cl^-, Mg^{2+}, K^+, Ca^{2+}, and total CO_2 in blood plasma of rainbow trout transferred from freshwater (FW) to 70% seawater (SW) and at 24 and 72 h following transfer to SW (n≥6). * indicates significant difference ($p<0.05$) from FW value.

	FW	24 h	72 h	SW
Cl^-	142.55±4.77	153.30±20.06	140.98±9.88	137.49±9.18
Na^+	153.98±4.21	142.63±11.7	160.17±6.59	159.88±6.28
Mg^{2+}	0.72±0.08	1.33±0.22*	0.80±0.08	0.74±0.07
Ca^{2+}	2.98±0.18	3.21±0.27	3.18±0.19	2.63±0.22
K^+	4.37±0.84	3.82±0.81	3.45±0.58	3.76±0.33
tCO_2	8.11±0.28	8.26±0.37	8.37±0.57	8.73±0.43

Figure 4.1. Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, K^+, Ca^{2+}, and tCO_2 of intestinal fluid spot samples from trout acclimated to 70% seawater (SW), and at 24 and 72 h following transfer from freshwater to SW (n=6-11). Different letters represent significant difference ($P<0.05$) between those times; shared letters or no letters indicate no difference.

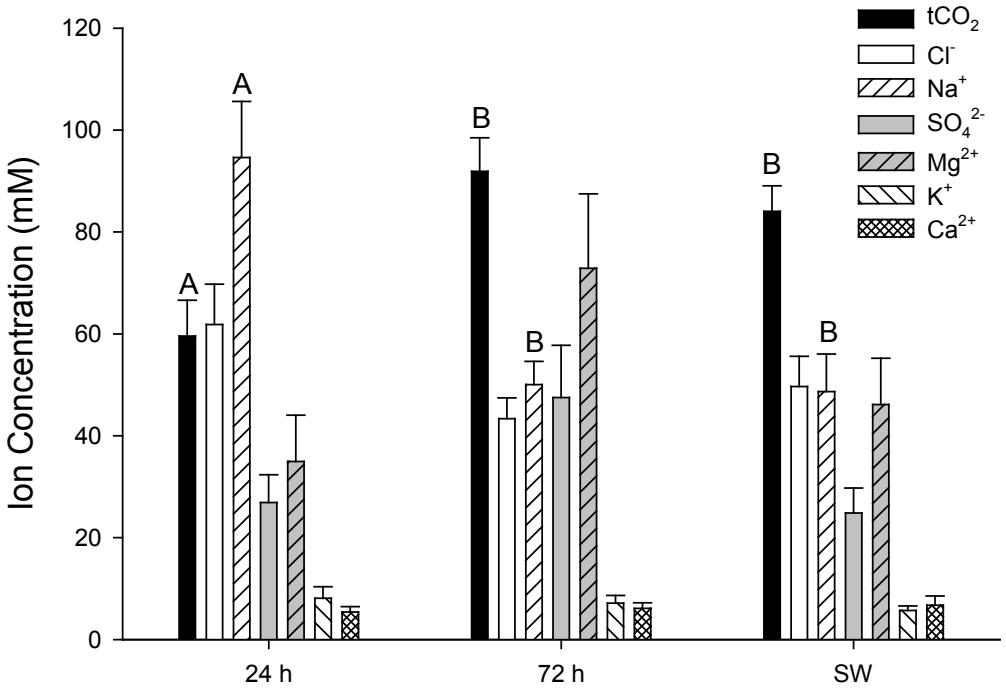


Figure 4.2. Flux of A) water (n=8), and B) tCO_2 ($n \geq 7$) in isolated intestinal sac preparations from trout acclimated to freshwater (FW), 70% seawater (SW), or 24 and 72 h following transfer to SW.

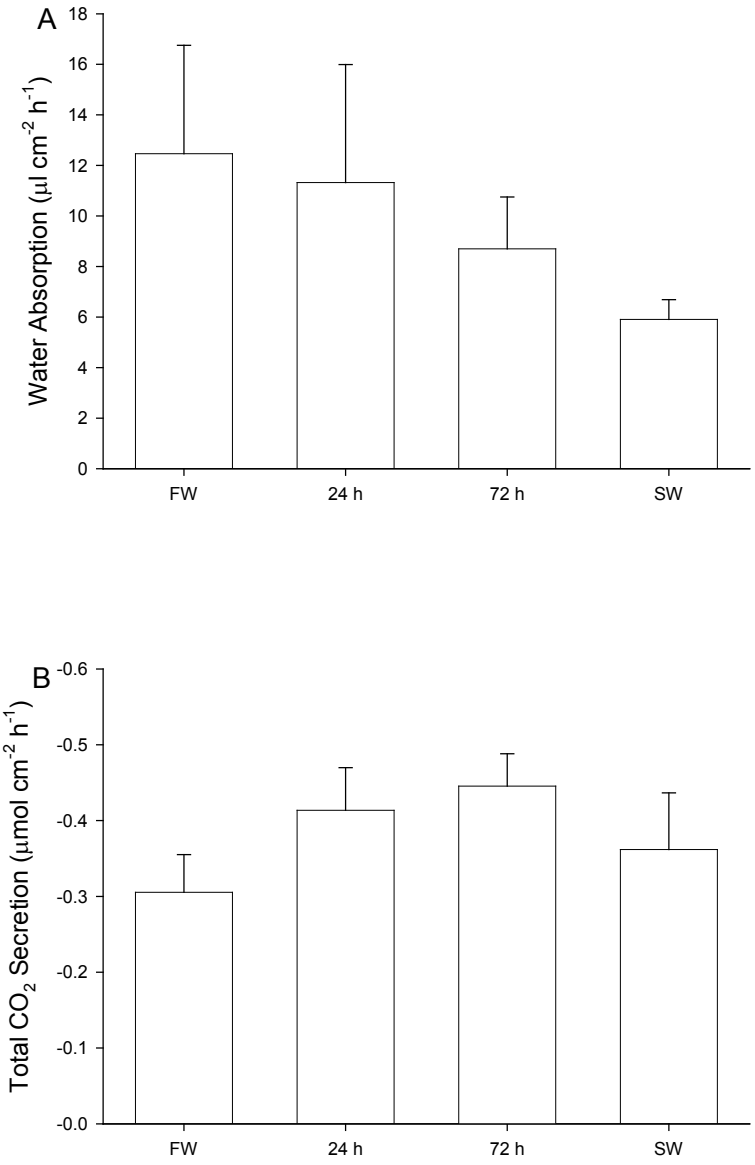


Figure 4.3. Flux of Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, K^+, and Ca^{2+} in isolated intestinal sac preparations from trout acclimated to freshwater (FW), 70% seawater (SW), or 24 and 72 h following transfer to SW. (n=5-8). Positive values indicate absorption; negative values indicate secretion.

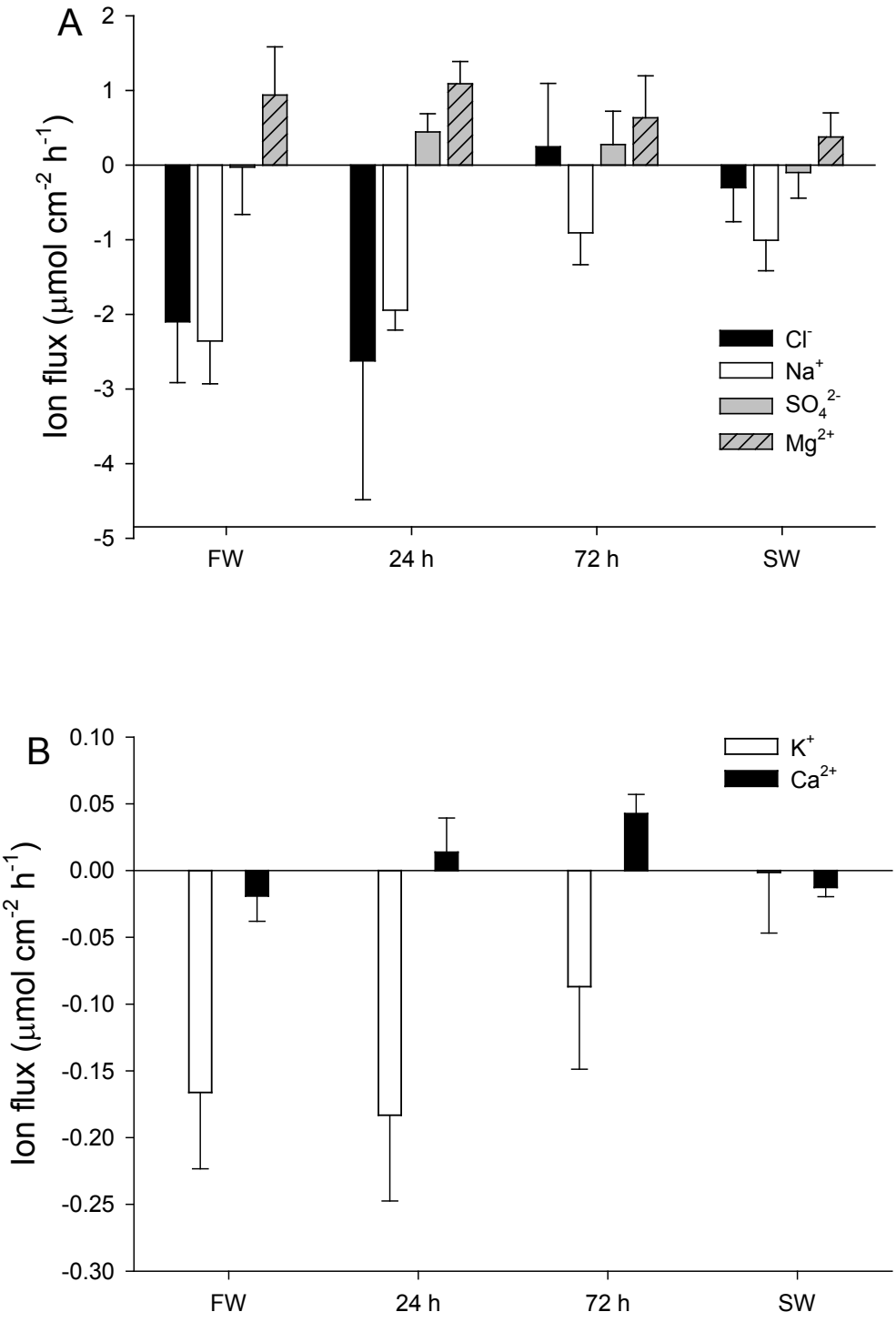


Figure 4.4. Secretion of base equivalents (μeqv cm^{-2} h^{-1}) of intestinal tissue from trout acclimated to freshwater (FW), 70% seawater (SW), and at 24 and 72 h following transfer to SW (n≥5). Different letters represent significant difference (P<0.05) between those times; shared letters indicate no difference.

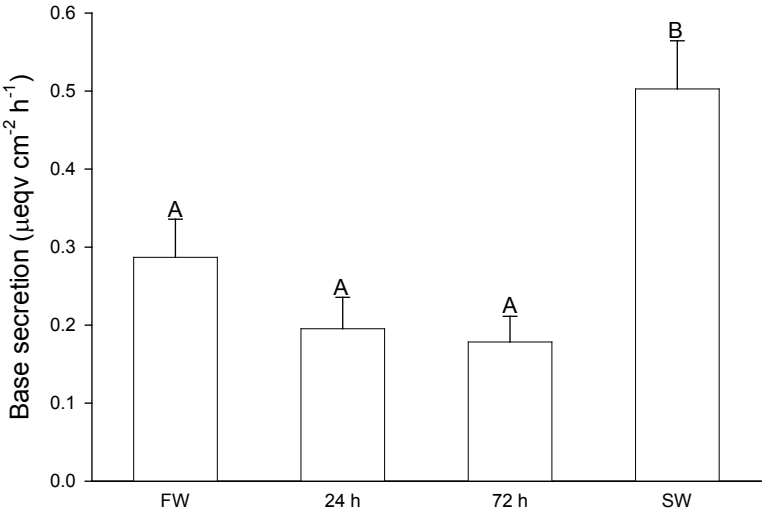


Table 4.5. Transepithelial potential (TEP, mV) and conductance (G, μSi cm^{-2}) of intestinal tissue from trout acclimated to freshwater (FW), 70% seawater (SW), and at 24 and 72 h following transfer to SW (n=5-6).

	TEP	G
FW	-8.89 ± 1.022	5.13 ± 0.262
24 h	-5.57 ± 1.315	4.79 ± 0.437
72 h	-9.84 ± 1.418	5.36 ± 0.211
SW	-9.98 ± 1.808	5.85 ± 0.154

Figure 4.5. Relative mRNA expression in trout acclimated to freshwater (FW) and 70% seawater (SW), and at 24 and 72 h following acute transfer to SW, determined by real-time qPCR and expressed relative to FW control, set to a relative value of 1 (dashed line). Relative expression, with samples without detectable expression removed from analysis, of A: vacuolar H^+-ATPase (V-ATPase) in pyloric cecae (n=7-8), and anterior intestine (n=2-7), and B: NBC1 in pyloric cecae. (n=7-8).

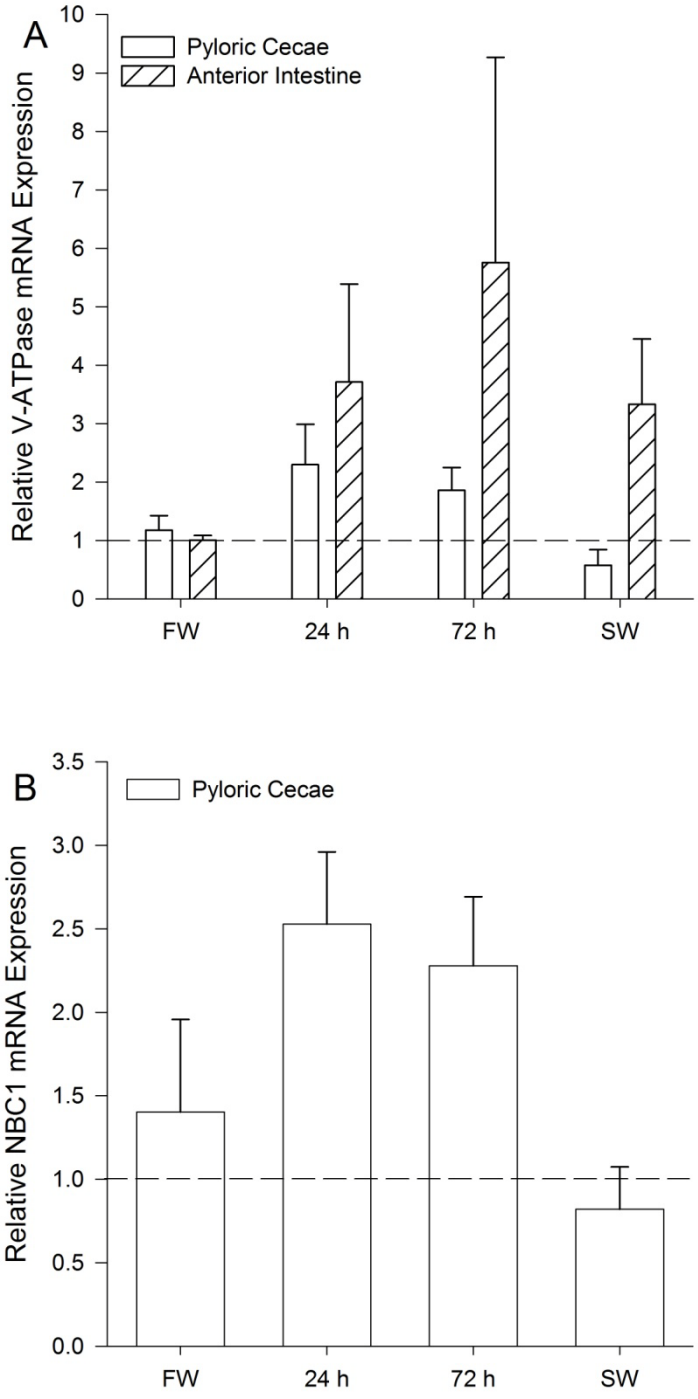


Figure 4.6. Relative expression of NHE2 mRNA in the gill of trout acclimated to freshwater (FW) and 70% seawater (SW), and at 24 and 72 h following acute transfer to SW, determined by real-time qPCR and expressed relative to FW control, set to a relative value of 1 (dashed line). (n=8).

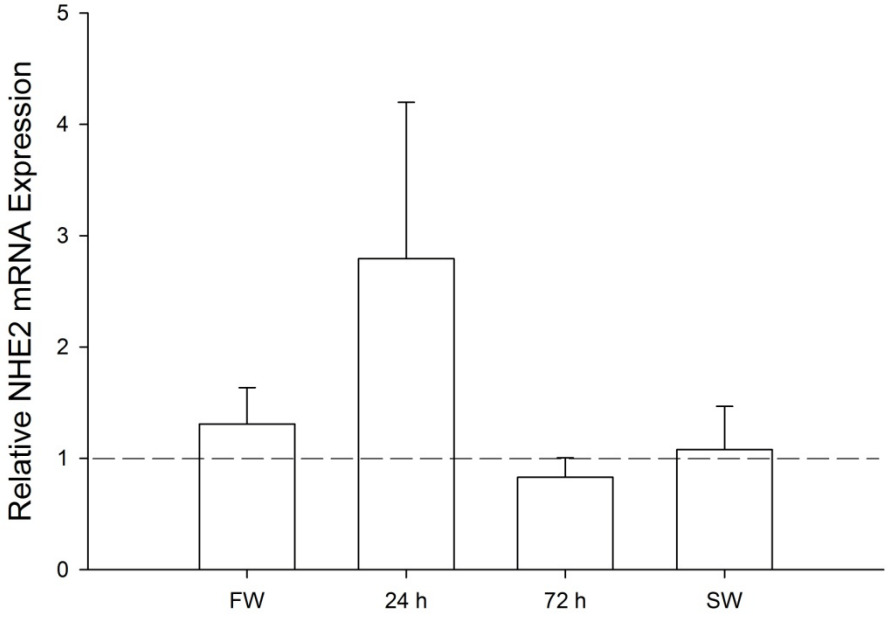


Figure 4.7. Relative expression of SLC26a6 mRNA in the kidney of trout acclimated to freshwater (FW) and 70% seawater (SW), and at 24 and 72 h following acute transfer to SW, determined by real-time qPCR and expressed relative to FW control, set to a relative value of 1 (dashed line). (n=7-8).

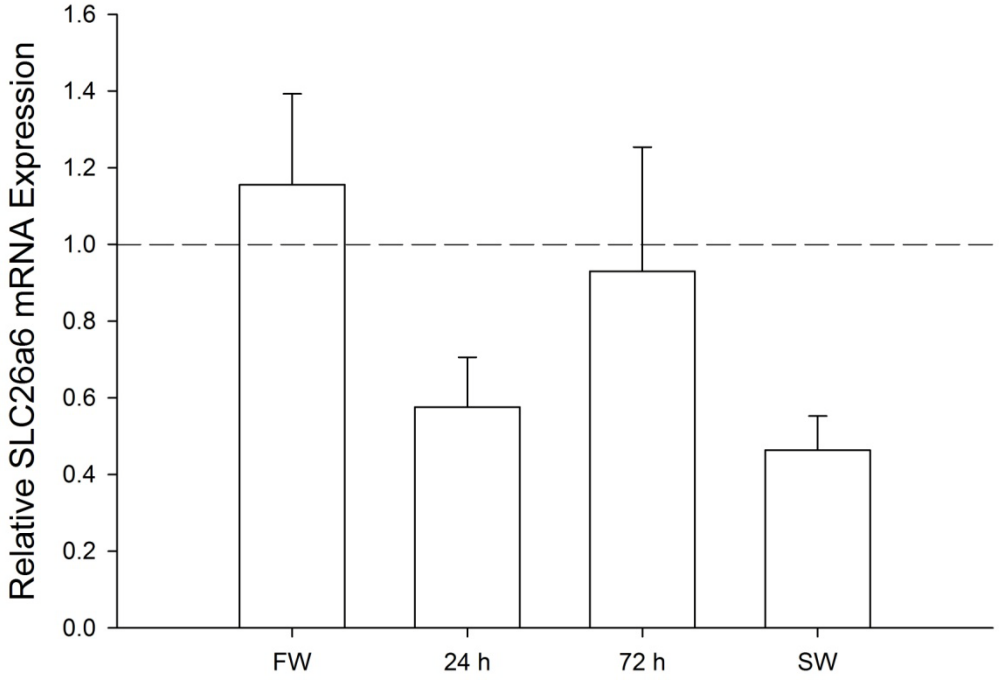
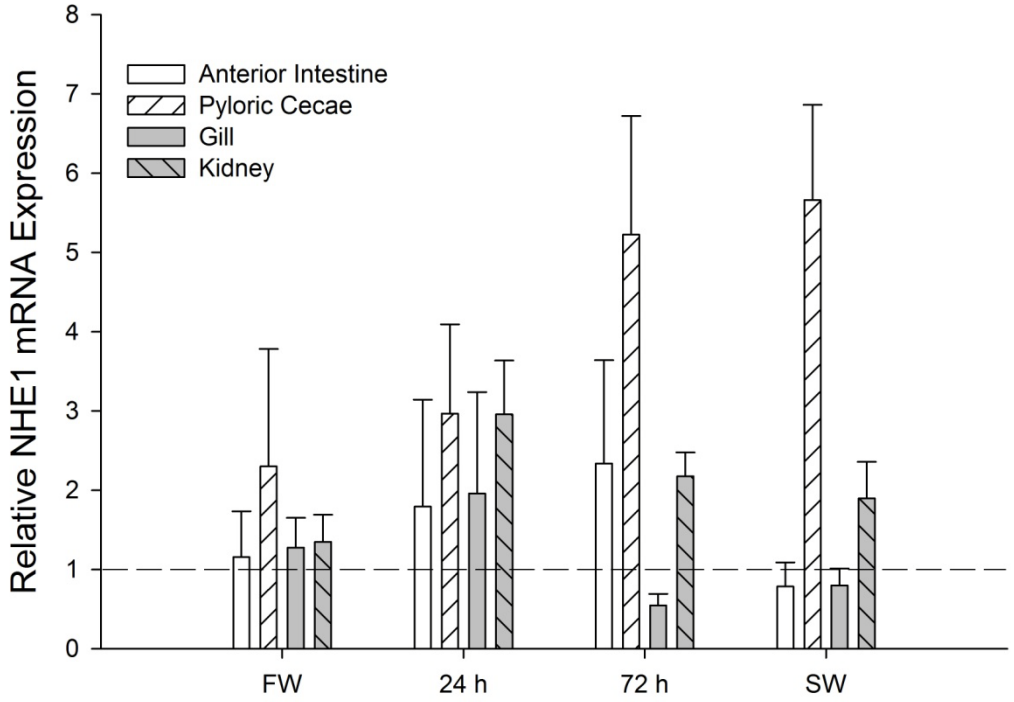


Figure 4.8. A: Relative mRNA expression, with samples without detectable expression removed from analysis, of NHE1 in the anterior intestine (n=2-8), pyloric cecae (n=4-8), gill (n=6-7), and kidney (n=7-8) of trout acclimated to freshwater (FW) and 70% seawater (SW), and at 24 and 72 h following acute transfer to SW, determined by real-time qPCR and expressed relative to FW control, set to a relative value of 1 (dashed line).



(Bath and Eddy, 1979; Grosell et al., 2007), and changes in ion composition of plasma and intestinal fluids occur within a relatively short time frame, peaking at ~24 h. Following these primary changes controlling osmo- and ionoregulatory demands, ion concentrations then stabilize over the next several days, but remain elevated over FW values (Leray et al., 1981; Grosell et al., 2007). The trout in this study did not follow this pattern, perhaps suggesting that they experienced less physiological stress due to acute salinity transfer than seen in previous studies, but explanations for why this might be are not readily apparent.

The small sample volumes of luminal fluids collected from FW-acclimated trout precluded measurements of tCO_2 and ion concentrations in these samples. The small volumes can be accounted for by the fact that FW-acclimated fish, unlike SW fish, have no need to drink. Trout were clearly impacted by the salinity change, as tCO_2 in the luminal fluid increased in SW-acclimated fish and at 72 h post-transfer, indicating increased absorption of Cl^-, and thus water. Similarly, while Na^+ in the luminal fluids remained relatively high at 24 h, the decreased Na^+ concentrations at 72h and in fish fully acclimated to SW were indicative of increased intestinal uptake (Fig. 4.1) and thus higher water absorption, a requirement for SW fish. These findings seem to suggest that the time between 24 and 72 h post-transfer is when transitional physiological adjustments to SW occur in the intestine. Given these changes occurring in the gastrointestinal tract, one of the most important osmoregulatory tissues in SW fish and one that is responsible for a large portion of the necessary ion and water uptake, these transport processes were studied in greater detail via isolation of the intestine, allowing for greater control of the conditions affecting ion and water flux.

Intestinal sac preparations

The *in vivo* effect of SW transfer was not reflected consistently in the intestinal sac preparations. These differences may be due to factors such as lack of perfusion, and endocrine and neurological controls, which influence the tissue *in vivo*, but not in the isolated tissue sacs. Additionally, the salines used for the *in vitro* experiments were designed to mimic luminal and extracellular fluids from SW fish (Table 4.1), so conditions for tissue isolated from FW and time points following acute transfer were somewhat different than those occurring *in vivo* (Fig. 4.1, Table 4.4), as these conditions place emphasis on differences with regards to tissue capacity for ion flux, rather than chemical gradients. None of the changes observed in the transport of water or ions across the intestinal epithelium using sac preparations were significantly different.

However, at 72 h trout appeared to be approaching water transport rates more characteristic of trout fully acclimated to SW than to FW (Fig. 4.2A). Fluid absorption occurred in all isolated intestinal sacs, although it was expected to be lower in FW trout. This may be due to water absorption being regulated primarily by fluid ingestion (*i.e.* drinking rate), rather than the tissue itself, as the FW fish intestine is equally capable of water absorption as intestine from fish in SW. The trend toward lower water absorption in SW-acclimated trout may also be accounted for by the decreased absorption of monovalent ions. Sacs demonstrated less negative net flux of Na^+, Cl^-, and K^+ after SW transfer (Fig. 4.3A), indicating a (predicted) increase in ion absorption in SW, driving water uptake. However, the secretion of Na^+ and Cl^- into the intestinal lumen occurring simultaneously with water absorption was very unusual. Measurements of tCO_2 in the intestinal sac preparations suggested the intermediate time points had higher rates of base

secretion than in either fully-acclimated state (Fig. 4.2B), further indicating that this period encompasses dynamic adjustments to intestinal osmoregulatory processes. Fluid tCO_2 has been previously shown to increase by 24 h following transfer to 65% seawater and remain elevated in fish acclimated for 3 weeks to this salinity (Grosell et al., 2007).

pH-stat titrations

The apparent decrease in TEP at 24 h following transfer from FW to SW (Table 4.5) indicated a rapid initiation of ionoregulatory adjustments by the tissue upon exposure to elevated salinity. However, this was a transient effect, with TEP increasing again by 72 h. Contrary to the increased conductance that would be expected if the decreased TEP was due to an increase in total ion permeability of the tissue, the decreased TEP was accompanied by a slight decrease in conductance. This apparent contradiction could be accounted for if the decreased TEP was due to increased activity and/or abundance of particular ion transport carriers, rather than overall permeability of the epithelium. This temporary change in electrophysiology of the tissue may be an indication of a regulatory switch from transporters favored in FW conditions to those favored in SW. Indeed, although not significant, TEP and conductance seemed to establish new baseline values following the reductions at 24 h (Table 4.5). Reduced (more negative) TEP and increased conductance from levels observed in FW-acclimated trout would be in line with the expected increased requirement for transepithelial ionic transport in the intestine of SW-acclimated trout. The base secretion rates calculated from the pH-stat titration (Fig. 4.4) indicated that SW trout consistently had higher base secretion rates than FW trout,

which was predicted based on the greater need for intestinal water uptake driven by absorption of monovalent ions including Cl^-.

The increased base secretion in SW trout assessed by the pH-stat titration method differed from the constant secretion rate of tCO_2 in the intestinal sac preparations. This apparent discrepancy is probably accounted for by the greater precision of the pH-stat method which titrates secreted base in real time. This active titration of secreted base creates an important distinction between the methods: luminal pH was maintained at 7.8 in pH-stat experiments, whereas intestinal sacs accumulated base and became alkaline. This may impact the behavior of the isolated tissue with respect to base secretion. In particular, accumulated luminal base in the sac preparations may inhibit activity of the Cl^-/HCO_3^- exchanger, and/or induce activity of the apical H^+ pump, both of which would reduce observed net base secretion. Furthermore, we suggest that this potential impact of the method is more apparent in the SW and not the FW trout because HCO_3^- secretion is of greater importance in the intestinal osmoregulation of SW-acclimated fish.

Gene expression assessed by qPCR

The physiological data discussed above indicated the functional adjustments occurring in the intestinal epithelia in response to salinity transfer. In addition, the regulation of ion transporters important to the current intestinal transport model (Grosell et al., 2009a) were investigated in all three osmoregulatory organs (gill, kidney, gastrointestinal tract). Tissues were not perfused, so expression particularly in gills and kidney may be complicated by blood contamination, although this situation has previously been shown to not impact expression values of the H^+-ATPase in trout (Perry et al., 2000), and is

probably not a substantial concern in the present study. The time course of 72 h considered in this study bracketed the period of maximum expression changes observed previously for V-type H^+-ATPase and Na^+-HCO_3^- cotransporter 1 (NBC1) (Grosell et al., 2007). Expression of V-type H^+-ATPase and NBC1 was investigated to confirm that the strain of rainbow trout used for the present study responded to seawater transfer in a similar manner to those as previously described in response to transfer to 65% SW (Grosell et al., 2007). Indeed, changes of NBC1 and V-type ATPase expression were qualitatively similar to previous observations (Fig. 4.5), although the magnitude of the changes was less robust.

Na^+ and H^+ transport pathways: Expression of NHE and V-type H^+-ATPase

V-type H^+-ATPase is expressed in gill, kidney, and intestine in freshwater trout, and is important for regulation of acid-base balance at the gill (Perry et al., 2000). In addition, the apical V-type H^+-ATPase in FW fish gill is linked to transport of Na^+ by creating a high membrane potential which drives Na^+ absorption via a Na^+ channel (Lin et al., 1994; Marshall and Grosell, 2005; Evans et al., 2005). In seawater, which requires branchial extrusion rather than absorption of Na^+, the V-type H^+-ATPase is of less importance in the gill (Lin and Randall, 1993; Lin et al., 1994; Hawkings et al., 2004). Instead, Na^+ and H^+ transport may occur by electroneutral exchange of these ions in SW-acclimated fish, in particular via apical NHE2 (Claiborne et al., 1999; Tresguerres et al., 2005; Ivanis et al., 2008). NHE2 increased after 72 h of hypercapnic acidosis (Ivanis et al., 2008), and branchial acid excretion increases in fish with increased intestinal base secretion due to hypersalinity exposure (Chapter 2). This led us to hypothesize there would be an

increase in NHE2 expression in the gill following transfer to increased salinity. In addition to acid-base impacts, effects on NHE expression may be of particular importance in the high-Na^+ marine environment (as opposed to freshwater).

In seawater acclimated fish, Na^+ is actively absorbed in the intestine to drive water uptake. It is well established that a large portion of Na^+ uptake occurs via Na^+-K^+-$2Cl^-$ cotransport (NKCC) and Na^+-Cl^- cotransport (NCC) (Skadhauge, 1974; Frizzell et al., 1979; Musch et al., 1982). However, a transport system similar to the branchial Na^+ transport via electroneutral Na^+/H^+ exchange which occurs in marine fish may also be important to Na^+ absorption by the intestinal epithelium. These proteins may also perform the transport of H^+ produced by CA-catalyzed endogenous CO_2 hydration in intestinal epithelial cells. While the presence of an apical H^+-ATPase has been demonstrated in the anterior intestine, this is not the case in the pyloric cecae (Grosell et al., 2009a), indicating that the pyloric cecae may rely more heavily on Na^+/H^+ exchange. In the present study, V-type H^+-ATPase was expressed in both the anterior intestine and the pyloric cecae, and expression transiently increased in the latter following SW transfer (Fig. 4.5A). Na^+-dependent H^+ excretion across the basolateral membrane has been shown to be an essential parameter for preventing cellular acidification in toadfish intestinal epithelium (Grosell and Genz, 2006). Removal of H^+ from the cytosol may occur across the basolateral membrane via NHE1, or at the apical membrane via NHE2 and/or NHE3. In this study, NHE1 exhibited a decreasing trend in expression in the gill following SW transfer, while NHE2 appeared to increase at 24 h post-tranfer, highlighting the need for increased branchial H^+ extrusion in fish with increased drinking and intestinal base secretion (Chapter 2). Similarly, branchial NHE1 has been shown to

decrease following a metabolic acidosis in sculpin, while the apical NHE2 increased branchial H^+ extrusion (Claiborne et al., 1999). NHE1 in the gastrointestinal tract was predicted to increase in expression following SW transfer, but expression of NHE1 did not differ significantly with salinity transfer in the anterior intestine, pyloric cecae, or kidney although it was found to be expressed in all of these tissues. However, the trend toward increased expression with increased salinity in the pyloric cecae is of interest given that this tissue has been recently identified as secreting base at equivalent rates to the anterior intestine (Grosell et al., 2009a). The expression of NHE1, but not NHE2, in the pyloric cecae suggests that, unlike the anterior intestine, H^+ may be extruded in the pyloric cecae primarily via basolateral NHE1 rather than the apical NHE2 and the H^+-ATPase.

Cl^- and HCO_3^- transport pathways: Expression of NBC1 and SLC26a6

Intestinal transport of HCO_3^-, resulting from CA-catalyzed endogenous CO_2 hydration in intestinal epithelial cells, is potentially performed by SLC26a6. Furthermore, SLC26a6 transports HCO_3^- in exchange for Cl^-, which is actively absorbed in the intestine to drive water uptake. Intestinal Cl^-/HCO_3^- exchange occurs via the SLC26a6 transporter in gulf toadfish (Grosell et al., 2009b), and SLC26a6 has been identified as the probable apical Cl^-/HCO_3^- exchanger in the intestine of *Takifugu obscurus* (Kurita et al., 2008). Both toadfish and *T. obscurus* have also been shown to rely on basolateral Na^+-HCO_3^- cotransport for transepithelial base transport (Kurita et al., 2008; Taylor et al., 2010). Trout NBC1 has been determined to be homologous to NBC1in other vertebrates and is highly expressed in intestine, gill, and kidney (Perry et al., 2003a). Based on previous

studies that showed increased intestinal HCO_3^- secretion due to increased salinity in the gulf toadfish (Chapter 2), SLC26a6 and NBC1 were predicted to be upregulated in the trout intestine following transfer to increased salinity.

The absence of expression of SLC26a6 in the pyloric cecae or anterior intestine (Table 4.3) was surprising given its importance in both toadfish and mefugu (Kurita et al., 2008; Grosell et al., 2009b). The lack of expression of SLC26a6 suggests that it is either absent from the tissue in rainbow trout, or is unimportant for processes dealing with salinity stress. Surprisingly, while SLC26a6 was expressed in the kidney, it was not upregulated following transfer to SW (Fig. 4.7); in fact, there was a trend for decreased expression in SW-acclimated trout ($p<0.079$). SLC26a6 has also been shown to increase in expression 30-fold in the kidney of *T. obscurus* exposed to SW conditions (Kato et al., 2009), presumably as a SO_4^{2-} transporter. Additionally, renal expression of SLC26a6 is of interest in light of its role in mammalian ion transport (Sindic et al., 2007). The decreasing trend in expression suggested that the trout has anion exchangers other than SLC26a6 that perform renal SO_4^{2-} excretion. Many anion exchangers are capable of transporting SO_4^{2-}, particularly among the SLC13 (Markovich and Murer, 2004) and SLC26 gene families (Mount and Romero, 2004). Those most likely to be expressed renally in the SW trout include SLC13a1, SLC13a5, SLC26a1, SLC26a5, and SLC26a11, all of which were expressed in *T. obscurus* kidney in addition to SLC26a6 (Kato et al., 2009). SLC26a1 and SLC13a1 have been demonstrated to be involved in renal SO_4^{2-} uptake in eel acclimated to FW (Nakada et al., 2005), and the observed downregulation of SLC26a6 in the trout kidney following acute SW transfer could suggest a role for this protein in tubular absorption rather than secretion in this species.

NBC1 was expressed in all pyloric cecae tissue samples (Table 4.3), and appeared to be transiently increased at 24-72 h post-transfer (Fig. 4.5).

Overview

Rainbow trout (*O. mykiss*) undergo freshwater to seawater migration as part of their life history, and are thus a good model organism for investigations concerned with the physiology of osmoregulation in these two environments. This study considered the response of *O. mykiss* to acute transfer to 70% seawater (SW) by *in vivo* sampling, isolated intestinal tissue preparations, and mRNA expression of select ion transporters. Water absorption, increased tCO_2 secretion, and adjustments to TEP and conductance by the intestinal epithelium all suggested that physiological changes to the gastrointestinal tract in response to SW conditions occurred 24-72 h post-transfer. V-type H^+-ATPase and NBC1 displayed increased expression at 24 and 72 h following SW transfer, confirming previous observations in this species (Grosell et al., 2007). Na^+/H^+ exchange, which may be important to both ion transport and regulation of acid-base balance, was also considered. Apical NHE2 was expressed in the gill as predicted, but was not expressed in the gastrointestinal tract. In contrast, NHE1 was demonstrated to be expressed in all examined tissues. Expression of NHE1 was increased in the anterior intestine and pyloric cecae following transfer to SW, suggesting that this transporter may account for both Na^+ absorption and removal of endogenously-produced H^+ on the basolateral membrane of the intestinal epithelium. The transporter identified as the intestinal Cl^-/HCO_3^- exchanger in other teleost species was not expressed in the

gastrointestinal tract of *O. mykiss*, and appeared to be downregulated in the kidney following SW transfer, suggesting that trout accomplish intestinal HCO_3^- and renal SO_4^{2-} excretion via alternative transporters.

Chapter 5

Fundulus heteroclitus acutely transferred from seawater to high salinity require few adjustments to intestinal transport associated with osmoregulation

Summary

The common killifish, *Fundulus heteroclitus*, has historically been a favorite organism for the study of euryhalinity in teleost fish. Despite the species' large range of salinity tolerance, studies of osmoregulation in high salinity are rare, with most previous studies focused on fish transferred between freshwater and seawater. Similarly, while branchial transport properties have been studied extensively, there are relatively few studies investigating the role of the gut in osmoregulation in the killifish. This study sought to characterize the fluid and ion transport occurring in the gastrointestinal tract of killifish adapted to seawater, and furthermore to investigate the adjustments that occur to these mechanisms following acute transfer to high salinity (70 ppt). *In vivo* samples of blood plasma and intestinal fluids of seawater-acclimated killifish indicated absorption of Na^+, Cl^-, and water, the relative impermeability of the intestine to Mg^{2+} and SO_4^{2-}, and active secretion of HCO_3^- into the intestinal lumen. The details of these processes were investigated further using *in vitro* techniques of isolated intestinal sac preparations and an Ussing chamber pH-stat titration system. However, these methods were discovered to be of limited utility under physiologically relevant conditions due to tissue deterioration. Results that could be validly interpreted suggested that there are few changes to intestinal transport following transfer to high salinity, and that adjustments to epithelial permeability occur in the first 24 h post-transfer.

Background

One of the characteristics distinguishing the killifish (*Fundulus heteroclitus*) as an exceptional model organism is its extensive range of tolerance to environmental variables, including salinity. Nearly two-thirds of killifish species are found in regions where they are exposed to a natural range of salinities spanning freshwater to seawater to even higher salinities. In the lab, *F. heteroclitus* can be transferred from brackish water (29 ppt) to freshwater or up to 114 ppt seawater without mortality (Griffith, 1974). Furthermore, *F. heteroclitus* naturally inhabit coastal salt marshes in salinities ranging from 0.4 to 35 ppt (Griffith, 1974), and regularly experience large changes in salinity due to tidal fluctuations. Investigation of the response of killifish to the high end of their salinity tolerance range allows for better understanding of the regulatory systems that provide this species with its impressive environmental flexibility.

Although killifish are able to tolerate salinities much greater than SW, studies utilizing transfer to hypersalinity are relatively rare. Most previous studies of the responses to salinity change in this species focused primarily on the physiological changes which occur during transfers between FW and SW. In general, *F. heteroclitus* displays similar physiological effects of FW to SW transfer as other euryhaline fish, but these adjustments occur more rapidly than in other species. In killifish transferred from FW to SW, plasma [Na^+] and osmolality increase within the first 12-24 h (Jacob and Taylor, 1983; Marshall et al., 1999), and return to FW levels after 4-5 days of SW exposure. While branchial transporters are reasonably well-described for killifish in both FW and SW (Wood and Marshall, 1994), intestinal ion and water absorption has also been shown to be of great importance to ionic and osmoregulation in teleost fish (Grosell,

2006). Killifish use the strategy of being at the ready with respect to branchial ion transport in brackish water and seawater conditions. For example, CFTR and NKCC are redistributed to the opercular epithelial surface following transfer from FW to SW (Marshall et al., 2002b), rather than requiring transcription following salinity transfer, which would produce a substantially slower response. Both CFTR and NKCC are also expressed in enterocytes of both FW- and SW-acclimated killifish (Singer et al., 1998; Marshall et al., 2002a) and it is reasonable to speculate intestinal transport may also be readily available to respond to changes in ambient salinity. An earlier study on killifish employing transfer from brackish water to FW indicated no transcriptional changes of ion transporters (NKA, CA2, CFTR, NKCC2) in the gut (Scott et al., 2006). These results may not reflect a lack of importance of these transporters in the killifish intestine, but rather that small salinity changes are not likely to reveal transcriptional changes because fish in estuaries inhabit brackish water and are regularly exposed to either freshwater or seawater when the tide changes. Exposure to 70 ppt seawater was used in this study to test the limits of the intestinal ion transport system to gain a better understanding of the characteristics most important to intestinal osmoregulation following increases in salinity.

The small size of *F. heteroclitus* sometimes becomes a difficulty for physiological measurements. For example, killifish cannot be cannulated for blood sampling. One possible way to expand beyond general *in vivo* sampling and gain more detailed information is to use *in vitro* methods, and a technique commonly used for killifish is the isolated opercular epithelium preparation. Since it was first described (Karnaky et al., 1977) the opercular epithelium has been used in many *in vitro* studies to examine branchial transport by means of the Ussing chamber method (reviewed by Wood and

Marshall, 1994) . However, the killifish gastrointestinal tract has been examined *in vitro* in only a handful of studies, and always under symmetrical (short-circuit) conditions (Marshall et al., 2002a; Scott et al., 2006; Scott et al., 2008; Wood et al., 2010). Even the extensively studied opercular epithelium only rarely has been examined using SW-like asymmetrical conditions in killifish (Degnan and Zadunaisky, 1979; Pequeux et al., 1988). While useful for transport studies, symmetrical conditions are not physiologically relevant with respect to ionic and acid/base parameters. To our knowledge, this study is the first to expose isolated intestinal tissue from *F. heteroclitus* to salines that mimic the natural composition of extracellular and luminal fluids (*i.e.* asymmetrical conditions).

In this study, *in vivo* sampling, *in vitro* pH-stat titration coupled with Ussing chambers, and isolated intestinal sac preparations (under both standard symmetrical and more physiologically relevant asymmetrical conditions) were all used to examine hypo-osmoregulation in killifish.

Materials and Methods

Experimental Animals

Killifish (*Fundulus heteroclitus*) were purchased from Aquatic Research Organisms Ltd. (Hampton, NH), and held in 62 l aerated aquaria at a maximum density of 50 animals per tank, according to an approved University of Miami animal care protocol (IACUC #09-001). Fish were held for at least two weeks in either 35 ppt filtered seawater from Biscayne Bay (Bear Cut, 34-37 ppt, 22-26°C) or 70 ppt seawater (Instant Ocean sea salt added to seawater). Fish acclimated to 35 ppt seawater were fed daily and held in flow-through conditions, while fish acclimated to 70 ppt seawater were held in tanks circulated

with a biofilter pump with weekly water changes and fed every other day. All fish were fed approximately 2% of their body weight, but starved at least 24 h prior to sampling.

In vivo Sampling

Fish were sacrificed by a blow to the head. Blood was sampled by caudal puncture using a heparinized 22-gauge needle fitted to a 1 ml syringe (BD Syringe), and the sample immediately centrifuged to isolate the plasma. The gastrointestinal tract was exposed by a ventral incision, clamped at the anterior and posterior ends, removed from the body cavity, and the intestinal fluid was drained directly into a sample tube.

pH-stat General Experimental Protocol

Experiments performed using an Ussing chamber, pH-stat system used the following protocol, modified from Grosell and Genz (2006). Fish were killed by a blow to the head and a segment (approx. 0.5 cm) of the anterior intestine removed, cut longitudinally, and mounted on a holding slide (Physiologic Instruments, P2403) exposing 0.1 cm^2 tissue. The mounted tissue was then placed between two Ussing half-chambers (Physiologic Instruments, P4000) filled with 1.6 ml of appropriate saline circulated with the corresponding gas (Table 5.1). All salines were brought to room temperature and gassed at least 30 min prior to experimentation. The pH of the mucosal saline was monitored using a combination electrode (Radiometer, PHC4000.8), and secreted base was automatically titrated with 0.005 N HCl by a pH-stat titration manager system (Radiometer, TIM 854 or 856) to maintain the pH of the luminal chamber at 7.800. The rate of base secretion in μmol cm^{-2} h^{-1} was calculated using the rate of HCl addition.

Electrophysiology measurements were obtained by recording voltage using AcqKnowledge software (v.3.8.1) under current clamp conditions, with a 30 μAmp short-circuit pulse every 60 sec. Conductance was calculated as the current divided by the recorded transepithelial potential per cm^2 of exposed tissue. All treatments were run for 60 min, following an initial 90 min control period, except for salinity transfer and luminal bafilomycin experiments, which had a 60 min control period.

pH-stat Experiments

Killifish anterior intestinal tissue was prepared and assessed in the pH-stat system for 4 h of titration to determine tissue viability and typical base secretion rates under asymmetrical conditions. Several types of experiments were run on the pH-stat system to characterize transport of acid-base equivalents and ions by the intestinal epithelium. Buffered serosal saline without HCO_3^- was used to eliminate base transport across the basolateral membrane (particularly by Na^+-HCO_3^--cotransport, NBC). Thus the base secretion recorded under these conditions must be attributed to a HCO_3^- source endogenous to the epithelium (intracellular metabolic CO_2 hydration, as opposed to transepithelial transport). Possible mechanisms for removal of H^+ produced via intracellular CO_2 hydration were considered by adding bafilomycin ($2x10^{-6}$ M in 0.1% DMSO), a pharmacological inhibitor of H^+-ATPase, to the luminal chamber. Serosal saline with reduced [Na^+] (15 mM) was used to investigate the presence of Na^+-dependent transporters, particularly Na^+-H^+ exchangers (NHE) and/or NBC, on the basolateral membrane. Finally, the effect of salinity transfer on base secretion of the epithelium was also investigated by using tissue isolated from fish fully acclimated to 35

ppt and 70 ppt seawater, and at 24 and 48 h following acute transfer to from 35 ppt to 70 ppt seawater.

Intestinal Sac Preparations

Intestinal sac preparations were used to examine flux of water, base, and ions across the intestinal epithelium following a protocol modified from Scott et al. (2006). The entire intestine except for the extremely fragile distal segment (last 10% of tract) was isolated by dissection as described above, and a filling catheter of PE50 tubing heat-flared on one end was inserted into the anterior end of the tissue and tied in place using 3-0 silk sutures. The sac was rinsed with 5 ml of mucosal saline (Table 5.1) and tied closed at the distal end. The preparation was then filled with mucosal saline containing 0.25 μCi ml^{-1} polyethylene glycol ^{14}C-4000 (PEG, specific activity 13.0 mCi g^{-1}), the filling catheter was heat-sealed, and the volume of saline precisely determined by weighing the Hamilton syringe before and after filling. For symmetrical sacs, the rinsing and filling saline was serosal saline without glucose; osmotic pressure was adjusted with mannitol to be equivalent to the serosal saline containing glucose. After filling, the sac was blotted dry, weighed, and placed in a 15 ml Falcon tube filled with 6 ml serosal saline (Table 5.1) gassed with 0.3% CO_2 in O_2. All serosal salines were gassed with this mix at least 30 min prior to the beginning of the flux to ensure correct pH. Following a 2 h flux period, the luminal saline was collected by cutting open the preparation. The tissue was then blotted dry and weighed. The filling catheter and sutures were removed and the tissue was spread out and traced onto paper to determine its surface area, with the weight of the traced area corrected to the weight of 1 cm^2 paper. Samples (1 ml) of serosal saline were

collected at the beginning and end of the flux period. The radioactivity of samples of the initial saline and saline collected before and after the flux period was measured using a Packard liquid scintillation analyzer (Tri-Carb 2100TR), following the protocol described in Grosell and Taylor (2007).

Sample Analysis and Calculations

Blood plasma, intestinal fluids, and luminal saline samples from intestinal sac preparations were all analyzed using the same methods. Total CO_2 was measured with a Corning 965 carbon dioxide analyzer. Samples were measured for Na^+, Mg^{2+}, K^+, and Ca^{2+} concentrations with fast sequential flame atomic absorption spectrometry (Varian 220, Palo Alto, CA, US) using an air/acetylene flame, and concentrations of Cl^- and SO_4^{2-} by anion chromatography (Dionex 120, Sunnyvale, CA, US).

^{14}C PEG-4000 was used as a tracer to determine water movement across the tissue. Water flux was calculated as the difference in concentration of ^{14}C PEG between initial and final luminal samples per cm^2 tissue per hour (Grosell and Taylor, 2007). Briefly, the initial volume was calculated as the volume of saline used to fill the sac preparation, corrected for dilution of the filling saline by mixing with fluid contained within the lumen during the filling process. The final volume was calculated as the concentration ratio of ^{14}C PEG of the initial saline and the final saline, multiplied by the initial volume. All radioactivity measurements were corrected for instrument background. Intestinal tissue was slightly permeable to ^{14}C PEG (< 8% error), as assessed by the appearance of ^{14}C in final serosal samples, and this was taken into account in water flux calculations by adding the radioactivity which crossed the tissue

over the course of the 2 h flux to the final luminal measurements. Flux of ions across intestinal sac preparations was calculated as the difference between the initial and final amounts of ion (μmol) in the lumen per cm^2 tissue per hour. Base equivalents were first calculated from pH and tCO_2 using the Henderson-Hasselbalch equation, as in Grosell *et al.* (2005), using a pK of 9.46, and then the flux was calculated in the same way as for the ions.

Statistical Analysis

All values are reported as the mean ± SEM. Student's t-tests were used to compare tCO_2 concentrations in intestinal fluids and intestinal sacs fluxes between 35 and 70 ppt seawater-acclimated fish. Individual differences for the pH-stat base secretion, TEP, and conductance were compared using paired Student's t-tests against the mean of the last 30 min of the control period. Differences were considered significant at $P<0.05$.

Results

In vivo samples

Blood plasma and intestinal fluids were sampled from fish acclimated to 35 ppt seawater (Table 5.2). In 35 ppt seawater-acclimated fish, K^+ and Ca^{2+} concentrations did not differ between the extracellular and intestinal fluids. Concentrations of Na^+ and Cl^- were higher in the plasma than the intestinal lumen. Mg^{2+} in the intestinal fluids was concentrated 100-fold over plasma concentrations and intestinal [SO_4^{2-}] was also high, with non-detectable levels in the plasma. Similarly, tCO_2 was highly concentrated in the intestinal

Table 5.1. Concentration of ions (mM) composing mucosal and serosal salines used in pH-stat and intestinal sac preparation experiments. Osmotic pressure was adjusted when necessary with mannitol, osmolality of serosal salines was measured without glucose. pH of serosal salines was adjusted to 7.8 with either HCl (regular and reduced Na^+) or NaOH (HCO_3^--free).

	Mucosal	Serosal	HCO_3^--Free	Reduced Na^+
NaCl	69.0	144.1	144.1	-
KCl	5.0	5.1	5.1	5.1
$CaCl_2 \cdot 2H_2O$	5.0	1.6	1.6	1.6
$MgSO_4 \cdot 7H_2O$	72.5	0.9	0.9	0.9
$NaHCO_3$	-	11.9	-	11.9
NaH_2PO_4	-	2.9	2.9	2.9
$MgCl_2 \cdot 6H_2O$	22.5	-	-	-
Glucose	-	5.5	5.5	5.5
N-methyl-D-glucamine	-	-	-	144.0
HEPES (Free Acid)	-	-	2.5	-
HEPES (Na^+-Salt)	-	-	2.5	-
mOsm	296	292	291	293
Gas	O_2	0.3% CO_2 in O_2	O_2	0.3% CO_2 in O_2

Table 5.2. Composition of blood plasma (n=9) and intestinal fluids (n=6) of killifish acclimated to 35 ppt seawater.

	Plasma	Intestinal Fluids
K^+	5.75±0.563	6.72±1.261
Ca^{2+}	13.91±0.825	15.15±2.020
Mg^{2+}	1.49±0.107	150.56±6.433
Na^+	137.92±1.069	41.16±3.818
Cl^-	145.90±5.668	97.39±7.552
SO_4^{2-}	--	87.24±4.153
Total CO_2	--	68.65±4.366

lumen of fish acclimated to 35 ppt seawater, as well as 70 ppt seawater-acclimated fish. Mean luminal tCO_2 (82.13±12.80) tended to be higher in fish acclimated to 70 ppt seawater than in 35 ppt seawater-acclimated fish (Table 5.2, 68.7±4.4), but this difference was not significant.

pH-stat

Assessment of the viability of the intestinal tissue was expected to be similar to what has been previously demonstrated in other fish species (Grosell and Genz, 2006; Fuentes et al., 2006; Grosell et al., 2009a). However, the base secretion (Fig. 5.1A) and conductance (Fig. 5.1C) of intestinal tissue exposed to asymmetrical conditions increased steadily over the course of 4 h of titration.

The experiment using HCO_3^--free serosal saline showed a maximum decrease in base secretion of 48% at 155 min (Fig. 5.2); base secretion recovered back to control levels after the treatment saline was replaced with regular serosal saline. Although, as with the viability experiment, the conductance was steadily increasing throughout the control and recovery periods, there was a noticeable variation during the HCO_3^--free serosal exposure which reduced the conductance from the upward-trending baseline levels. There also appeared to be a reduction in TEP during the HCO_3^--free exposure, and the TEP increased back toward control levels during the recovery period.

The serosal saline with greatly reduced $[Na^+]$ impacted the electrophysiology of the tissue as expected, with dramatically increased TEP and decreased conductance (Fig. 5.3B and C). Surprisingly, base secretion by the intestinal epithelium increased under low Na^+ conditions (Fig. 5.3A).

Killifish acutely transferred from 35 to 70 ppt seawater experienced few changes to intestinal transport following transfer. Assessment using two-way ANOVA of HCO_3^- secretion indicated no effect of time following transfer from 35 ppt to 70 ppt seawater (Fig. 5.4). The only significant time effect was a reduction in TEP (Fig. 5.5) at 24 h following transfer to 70 ppt seawater. Conductance increased slightly at 24 h post-transfer (Fig. 5.6), but this change was not significant (P<0.081). Fish acclimated to 70 ppt seawater and at 48 h post-transfer exhibited electrophysiological characteristics not significantly different than those of the 35 ppt seawater-acclimated fish.

Addition of bafilomycin to the mucosal saline significantly increased base secretion, with the most robust change occurring in fish sampled 24 h post-transfer. Although conductance of the tissue also increased significantly following bafilomycin addition at all time points (Fig. 5.6), this cannot be interpreted as a specific pharmacological effect, as viability experiments demonstrated increased conductance with increased time the tissue was in the system (Fig. 5.1). No change in TEP further supported the lack of an effect of luminal bafilomycin on the electrophysiology of the tissue. Sham-transfer controls for handling stress in the salinity-transferred fish were not significantly different than fish acclimated to 35 ppt seawater used as bafilomycin controls (data not shown).

Intestinal sac preparations

Isolated sac preparations under symmetrical (serosal saline on both sides) conditions absorbed water and secreted base as is expected for intestinal tissue from marine fish. However, sacs with asymmetrical conditions exhibited negative water flux (water was

lost into the lumen of the sac) (Fig. 5.7). Base flux (Fig. 5.8) was negative in both sets of sacs, indicating the expected secretion of HCO_3^- from the serosal to the mucosal side of the epithelium. The rate of base secretion occurring in the asymmetrical sacs was very similar to that of the symmetrical sacs. There were no changes to the flux of ions in the asymmetrical sacs (Fig. 5.8B, Table 5.3) between fish acclimated to 35 or 70 ppt seawater. The symmetrical sacs displayed significantly decreased flux of Mg^{2+} and Ca^{2+} in 70 ppt seawater (Table 5.3); there were no other changes in ion flux. Flux of water, Na^+, and Cl^- differed significantly between the symmetrical and asymmetrical sacs, regardless of the acclimation salinity.

Conclusions

Plasma and intestinal fluids sampled from SW-acclimated killifish demonstrated chemical composition similar to that of other marine teleost species. Increased concentrations of Na^+ and Cl^- in the plasma were indicative of the active absorption of these ions from the intestinal lumen, while highly concentrated luminal Mg^{2+} and SO_4^{2-} suggest substantial water absorption across the intestinal epithelia. Similarly, high tCO_2 suggested active exchange of Cl^-/HCO_3^- was also occurring, driving water uptake and increasing tCO_2 in the lumen. The increase of tCO_2 in killifish acclimated to 70 ppt seawater may indicate a greater role for this transport mechanism in higher salinity.

Figure 5.1. A: Base secretion (μequiv cm^{-2} h^{-1}), B: transepithelial potential (mV), and C: conductance (μSi) of isolated anterior intestinal tissue over 4 h of base secretion with serosal saline changed at 2 h. * indicates significant difference from the mean of the last 30 min of the control period (black). n=5-6.

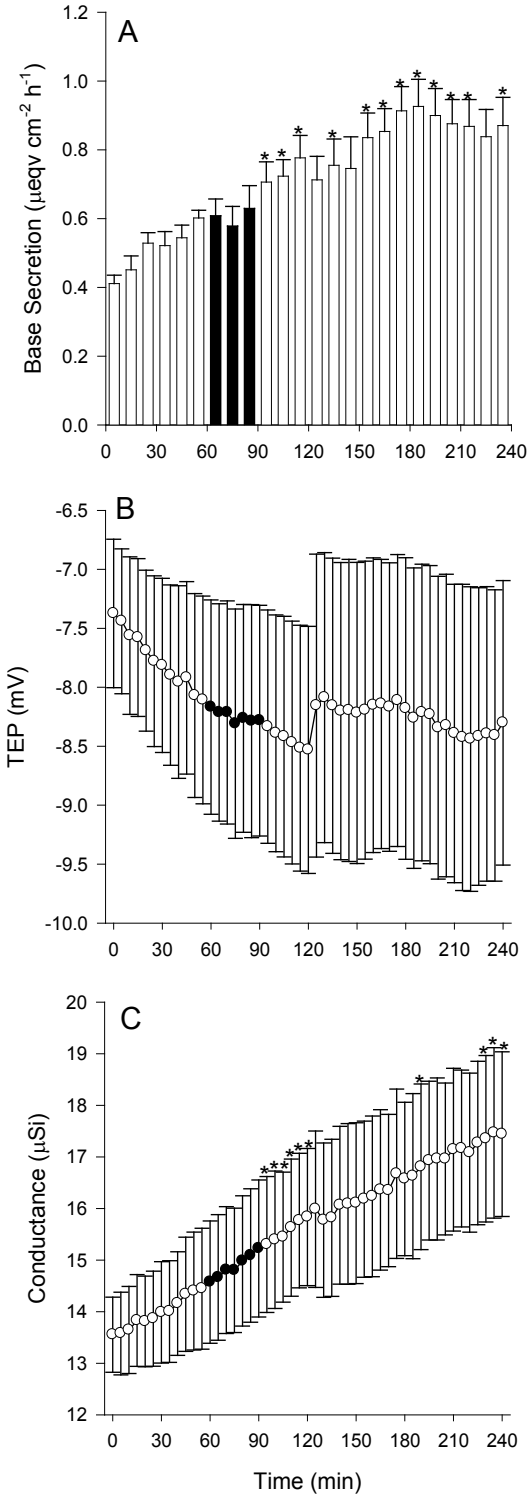


Figure 5.2. A: Base secretion (µequiv cm^{-2} h^{-1}), B: transepithelial potential (mV), and C: conductance (µSi) of isolated anterior intestinal tissue exposed to serosal saline lacking bicarbonate (grey) following a 90 min control period with serosal saline of usual ionic composition (white). n=5. * indicates significant difference from the mean of the last 30 min of the control period (black).

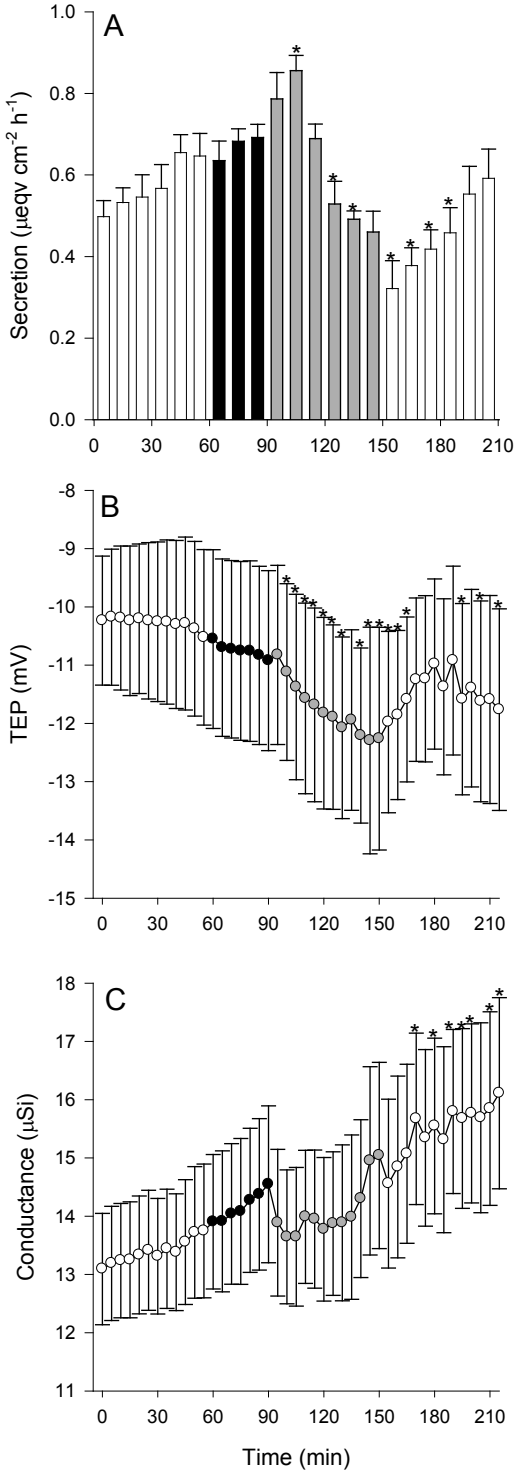


Figure 5.3. A: Base secretion (μequiv cm^{-2} h^{-1}), B: transepithelial potential (mV), and C: conductance (μSi) of isolated anterior intestinal tissue exposed to serosal saline with Na^+ reduced to 15 mM (grey) following a 90 min control period with serosal saline of usual ionic composition (white). n=5. * indicates significant difference from the mean of the last 30 min of the control period (black).

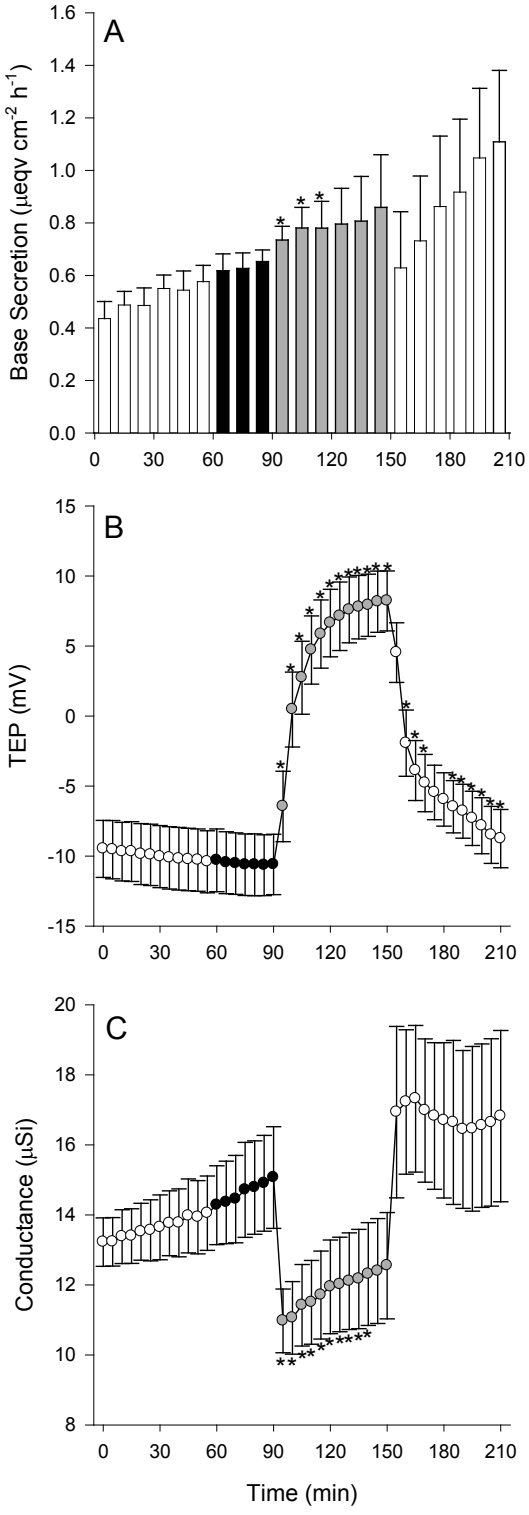


Figure 5.4. Base secretion (μequiv cm^{-2} h^{-1}) of isolated anterior intestinal tissue from fish acclimated to 35 ppt seawater, 70 ppt seawater, and at 24 and 48 h following acute transfer to 70 ppt seawater, followed by addition of bafilomycin to luminal saline (grey). n=5-8. * indicates significant difference from the mean of the last 30 min (black) of the 60 min control period (white).

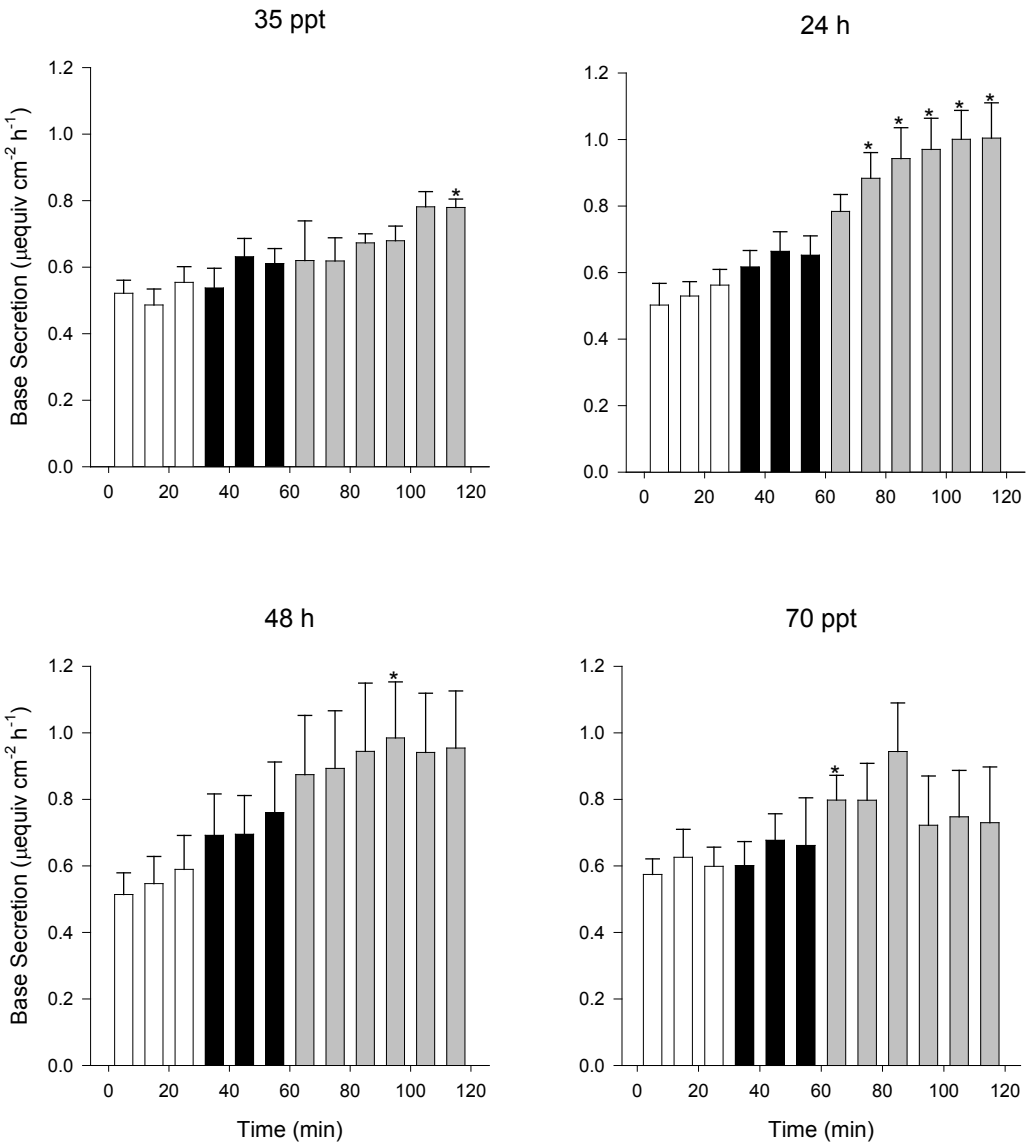


Figure 5.5. Transepithelial potential (mV) of isolated anterior intestinal tissue from fish acclimated to 35 ppt seawater, 70 ppt seawater, and at 24 and 48 h following acute transfer to 70 ppt seawater, followed by addition of bafilomycin to luminal saline (grey). n=5-8. * indicates significant difference from the mean of the last 30 min (black) of the 60 min control period (white).

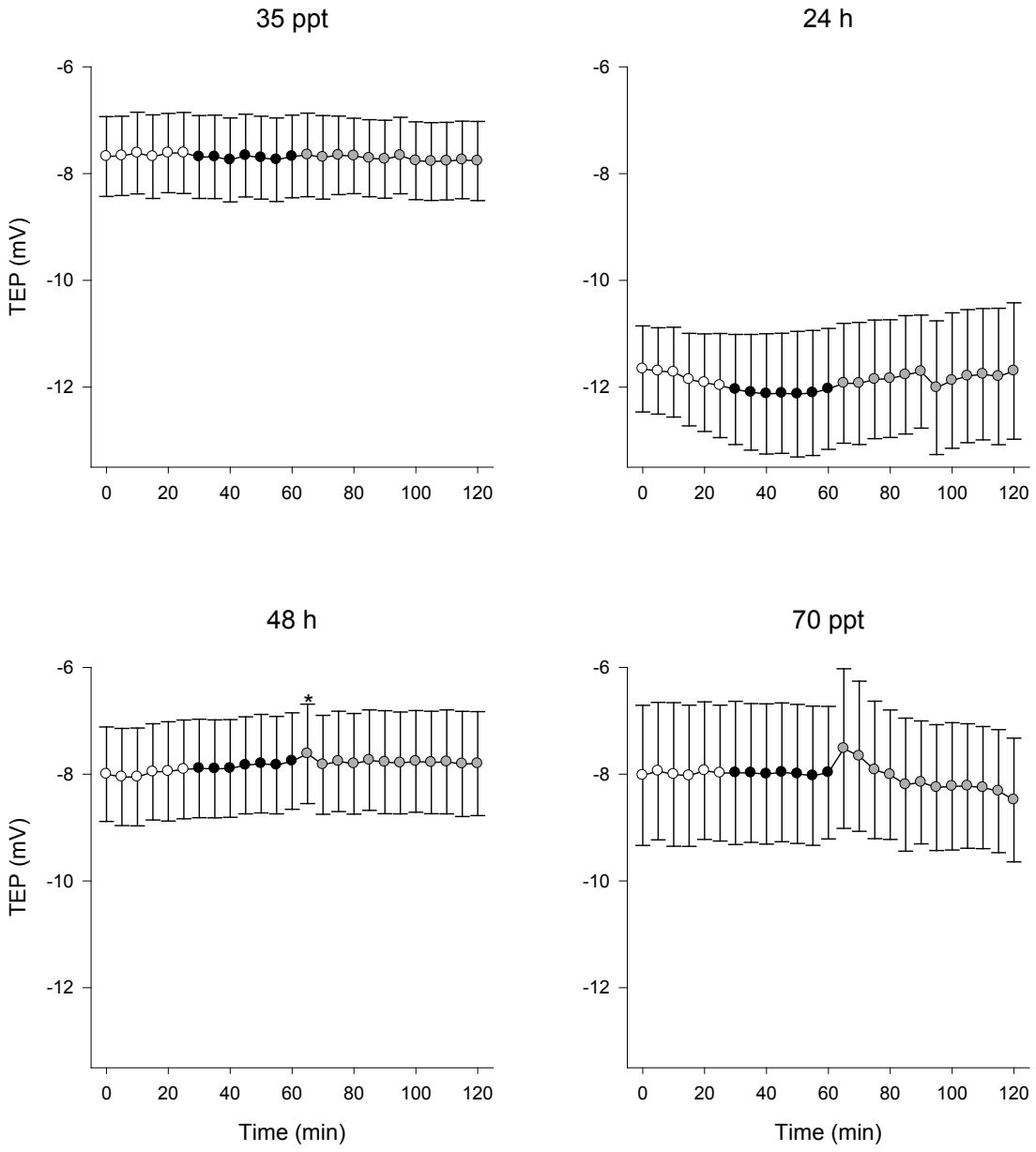


Figure 5.6. Conductance (μSi) of isolated anterior intestinal tissue from fish acclimated to 35 ppt seawater, 70 ppt seawater, and at 24 and 48 h following acute transfer to 70 ppt seawater, followed by addition of bafilomycin to luminal saline (grey). n=5-8.
* indicates significant difference from the mean of the last 30 min (black) of the 60 min control period (white).

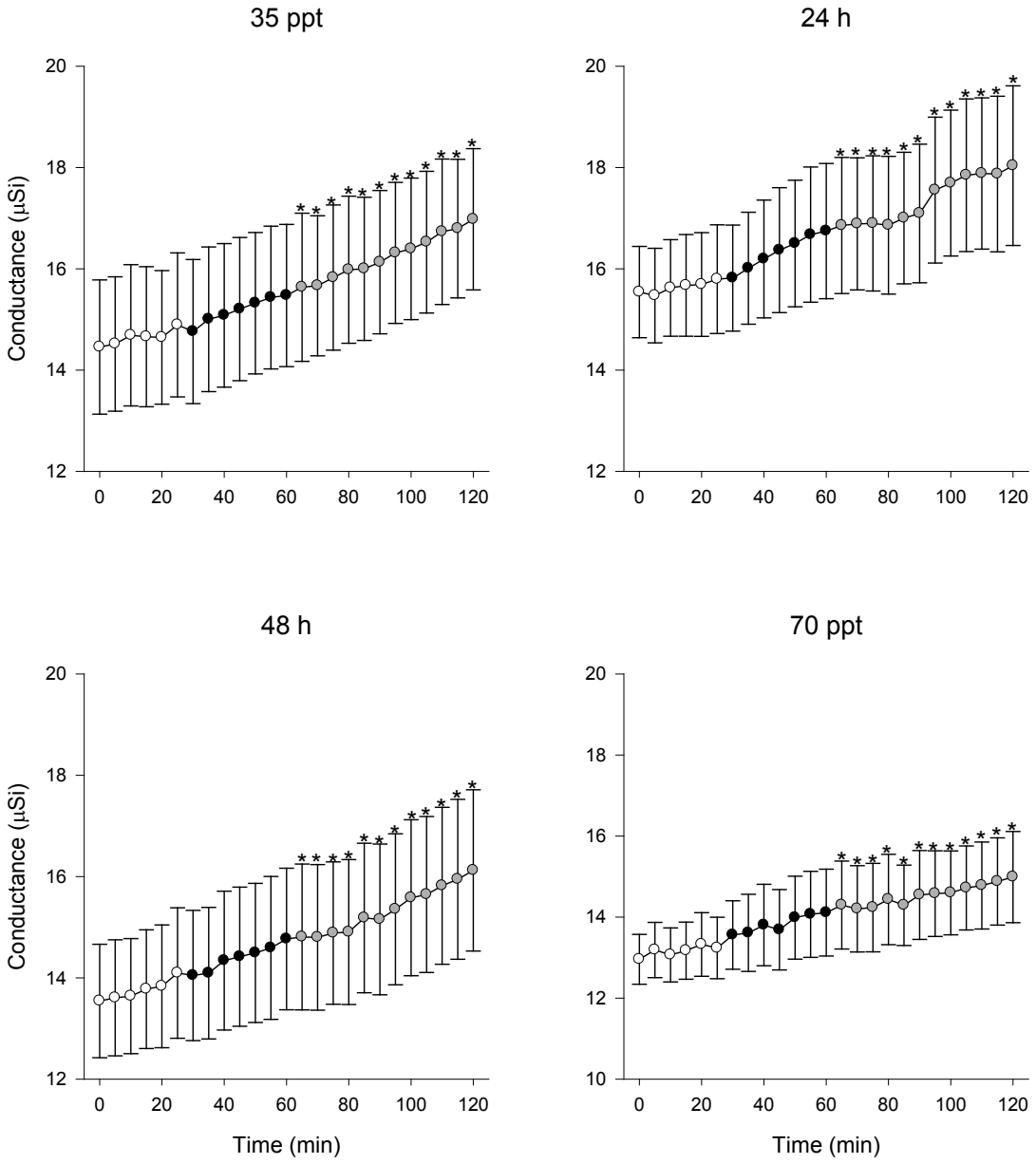


Figure 5.7. Flux of water (µl cm^{-2} h^{-1}) in the isolated intestinal epithelium from killifish acclimated to 35 and 70 ppt seawater in asymmetrical (black bars, n=8-9) and symmetrical (white bars, n=4-6) sac preparations.

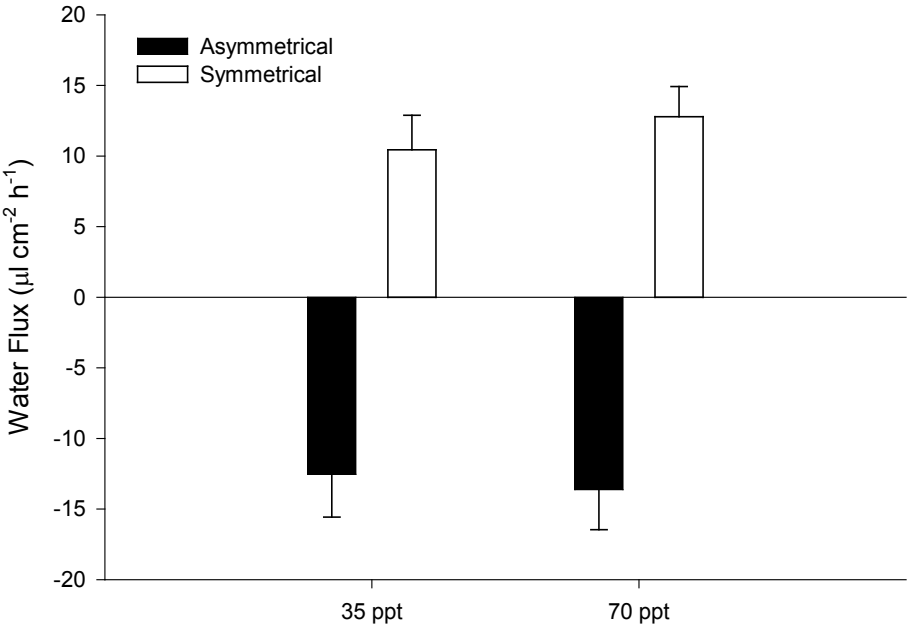
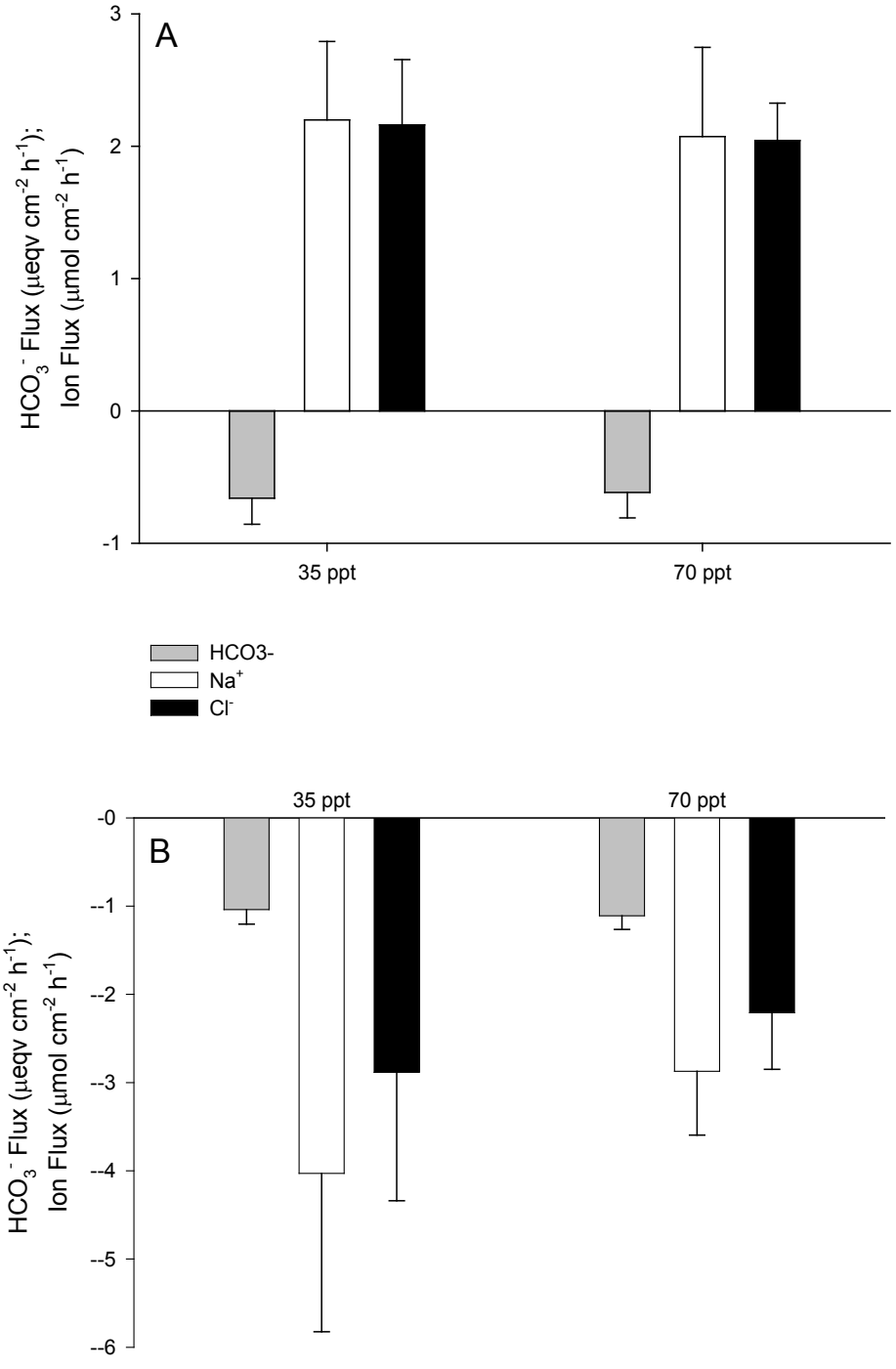


Table 5.3. Flux (µmol cm^{-2} h^{-1}) of Mg^{2+}, SO_4^{2-}, Ca^{2+}, and K^+ across the isolated intestinal epithelium from killifish acclimated to 35 and 70 ppt seawater in symmetrical (n=4-6) and asymmetrical (n=8-9) sac preparations. * indicates significant difference between 35 and 70 ppt seawater.

	Symmetrical		Asymmetrical	
	35 ppt	70 ppt	35 ppt	70 ppt
Mg^{2+}	-0.130±0.056	-0.003±0.011*	-0.412±0.113	-0.522±0.218
SO_4^{2-}	-0.058±0.023	-0.019±0.006	-0.551±0.166	-0.358±0.185
K^+	-0.069±0.034	-0.074±0.051	-0.226±0.108	-0.228±0.105
Ca^{2+}	-0.085±0.022	-0.005±0.013*	-0.051±0.009	-0.051±0.025

Figure 5.8. Flux of base equivalents (µequiv cm^{-2} h^{-1}), Na^+, and Cl^- (µmol cm^{-2} h^{-1}) across the isolated intestinal epithelium from killifish acclimated to 35 and 70 ppt seawater in A: symmetrical (n=4-6) and B: asymmetrical (n=8-9) sac preparations.



Previous investigations of killifish showed higher drinking rates in fish in brackish water over freshwater, and further increases upon transfer from brackish water to SW (Potts and Evans, 1967; Scott et al., 2006; Scott et al., 2008). In other teleost species, increased drinking following transfer to salinities higher than seawater is well established (Maetz and Skadhauge, 1968; Gonzalez et al., 2005). In addition, it has been demonstrated that gradual changes mimicking tidal salinity shifts result in less dramatic shifts to ion and fluid transport than those that occur following acute transfer, suggesting that killifish maintain osmoregulatory transport properties optimized for seawater exposure even when in freshwater conditions (Prodocimo et al., 2007; Wood and Grosell, 2008; Wood and Grosell, 2009). These observations on drinking rate in killifish are particularly important for intestinal osmoregulation, as the constancy of intestinal ion and water transport mechanisms, regardless of ambient conditions, makes ingestion of seawater (*i.e.* drinking rate) the main regulatory force for intestinal contribution to osmoregulation.

Intestinal sac preparations under symmetrical conditions absorbed water (Fig. 5.7) as expected based on previous studies (Wood et al., 2010). Under symmetrical conditions, net efflux of Mg^{2+} and Ca^{2+} were lower in 70 ppt than 35 ppt seawater (Table 5.3), and SO_4^{2-} efflux also decreased, although this trend was not significantly different. Killifish in high salinity may therefore have decreased permeability of the epithelium to these ions and thus decreased need for secretion.

The ionic composition of the mucosal saline used in the asymmetrical sacs (Table 5.1) was reasonably similar to the intestinal fluids sampled *in vivo* (Table 5.2); Na^+ and Cl^- were higher than *in vivo* measurements, while Mg^{2+}, SO_4^{2-}, Ca^{2+}, and K^+

concentrations were slightly lower in the saline than *in vivo*. These conditions should favor absorption of Na^+, Cl^-, and water if the tissue were behaving as it does *in vivo*. However, preparations under asymmetrical, *in vivo*-like conditions lost water, Na, and Cl^- (*i.e.* transport into the lumen), and this anomalous behavior must be attributed to inhibition of normal ionic transport in the intestinal tissue. Furthermore, it has been shown that intestinal epithelia exposed to flux periods longer than 2 h experience isotopic recycling (Scott et al., 2006), suggesting that the isolated tissue is not robust even under symmetrical conditions.

In accordance with the anomalous results in the asymmetrical intestinal sac preparations, the viability of the isolated intestinal tissue in an Ussing chamber pH-stat titration system was less than that for other species examined so far (Fig. 5.1) (Grosell and Genz, 2006; Grosell et al., 2009a). The reason the intestinal tissue was not viable in the pH-stat system cannot be easily determined. One possibility is that the small surface area of mounted tissue (0.1 cm^2) compounds edge effects and does not expose enough undamaged tissue to the chamber for meaningful measurements to be recorded. However, exposure of the same surface area in this system with another delicate tissue, the pyloric cecae of rainbow trout, has been used successfully in a previous study (Grosell et al., 2009a), so edge effects probably do not account for the difficulty encountered using the killifish intestine. Another possibility is that the tissue simply cannot remain intact for long without supporting mechanisms (*e.g.* blood perfusion, endocrine controls, etc.) present *in vivo*. Viability data showed increasing base secretion and conductivity (Fig. 5.1A and C), making it difficult to interpret the results of the other experiments performed in this study using the Ussing chamber pH-stat titration system.

The changes in these parameters under baseline conditions indicated that, contrary to expectations, the intestinal tissue of killifish was not stable in the pH-stat system. Short time spans [such as the secretion (Fig. 5.1A) and TEP (Fig. 5.1B) measurements in the 30 min control period] do appear stable, as changes occur slowly, but the tissue was clearly not stable over the time required to run a meaningful experiment.

Several experiments intended to characterize the ionic transport occurring across the intestinal epithelium were performed prior to the discovery of the instability of the tissue in these isolated tissue preparations. However, some of the effects on the intestinal tissue are distinct enough to be apparent despite the complication imposed by the unstable baseline measurements. The results of these experiments using the pH-stat method are reported above, but must be interpreted cautiously. Statistical significance may not always be indicative of a treatment effect, but rather of the steady degeneration, as indicated by increased conductance (Fig. 5.1C), of the isolated tissue over time.

The results of the HCO_3^- free experiment were particularly obscured by the instability of the intestinal tissue in the Ussing chamber system. However, it did appear that secretion of base and epithelial conductance are both decreased after ~30 min exposure to HCO_3^- free serosal saline, and that the tissue took ~30 min more to recover to control base secretion rates. If this observation is accurate, it would suggest approximately half of the secreted base originates from the extracellular fluid, as previously indicated for other teleost species (Grosell and Genz, 2006).

The increase of base secretion under low Na^+ conditions (Fig. 5.3A) was very surprising, given the current model for intestinal ion transport related to HCO_3^- secretion predicts decreased HCO_3^- secretion due to decreased activity of important basolateral

transporters, specifically the Na^+-H^+ exchanger and the Na^+- HCO_3^- cotransporter, under low Na^+ conditions (Grosell and Genz, 2006; Grosell et al., 2009a; Taylor et al., 2010). However, the gradually increased rate of base secretion that occurred in the Ussing chamber system under control conditions (Fig. 5.1A) suggests this result may have been a methodological artifact, rather than a treatment effect. Alternatively, the effect may be due to the change from negative to positive TEP, as this would contribute an extra electrical gradient favoring Cl^- uptake, in exchange for HCO_3^- secretion.

Because tissues from each time point following transfer to 70 ppt seawater should behave similarly in the Ussing chamber set up, with respect to tissue deterioration, comparison among these groups is valid. Changes to electrophysiology at 24 h (Figs. 5.5 and 5.6) indicate that there were some adjustments occurring in the transport across the intestinal tissue, which were apparently completed by 48 h post-transfer. There was no effect of time on base transport (Fig. 5.4), indicating few dynamic changes to transport due to salinity transfer and that fish acclimated to 35 ppt seawater are always physiologically prepared to tolerate high salinity. Thus, the ion transport system driving water uptake in fish acclimated to 35 ppt seawater must respond to the greater osmoregulatory demand of 70 ppt seawater relatively quickly and easily.

Recent studies have suggested that intestinal Cl^-/HCO_3^- exchange may be coupled with an apical H^+-ATPase (Grosell et al., 2007; Grosell et al., 2009b). In agreement with this hypothesis, addition of the H^+-ATPase inhibitor bafilomycin to the luminal half-chamber increased net base secretion (Fig. 5.4) by the killifish anterior intestine. Furthermore, while base secretion did not differ with salinity transfer, there was a significant increase in base secretion with bafilomycin addition at all post-transfer time

points. At 24 h, this effect was particularly pronounced, as rates of base secretion were increased within 20 min after luminal addition of bafilomycin, and remained elevated for the duration of the treatment period. We therefore conclude that apical H^+ secretion was impacted by transfer to high salinity, and that this impact may be of particular interest in the first 24 h following acute transfer. Interestingly, this effect coincided with the observed changes to the electrophysiology of the epithelium post-transfer. It therefore appears that the principal changes in transport properties of the intestinal epithelium, including increased apical H^+ extrusion, occur at ~24 h following transfer to increased salinity.

Overview

Killifish are a common organism used for the characterization of euryhalinity in fish. Despite their high salinity tolerance, the physiology of the species under these conditions has been rarely investigated. This study considered intestinal transport mechanisms in hypo-osmoregulation of seawater killifish, and the role of the intestine to osmoregulation in killifish transferred to 70 ppt seawater. Transfer from 35 to 70 ppt seawater has little impact on the ion and water transport properties of killifish, suggesting that the osmoregulatory strategy employed by seawater-acclimated fish does not require alteration for the killifish to tolerate higher salinities. However, decreased viability of the intestinal tissue *in vitro* under asymmetrical conditions raises concerns about the physiological relevance of these types of measurements for this tissue. Even with these experimental limitations, we conclude that killifish acutely transferred to high salinity

require few alterations to intestinal osmoregulatory transport of water and ions leaving adjustments of drinking rate as a likely regulatory control. Alterations to the permeability of the tissue occur within the first 24 h post-transfer.

Chapter 6

Discussion

The Krogh principle is a basic tenet of comparative physiology, stating that "for a large number of problems, there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh, 1929; Krebs, 1975). This study sought to make use of the Krogh principle by using three fish species of varying natural salinity tolerance to understand the physiological mechanisms that exist to combat external challenges to maintenance of internal ion, water, and acid-base balance. Ecological factors drive research on the physiology of salinity tolerance in fish and this research in turn expands our understanding of the physiological constraints on vertebrate life in aquatic environments. Furthermore, teleost fish represent approximately half of all vertebrate species, and understanding regulatory processes, which determine physiological adaptation to variable environments, in fish may provide a baseline for research in other vertebrates, including mammals. Each research chapter comprising this dissertation expands the information available on this topic, as well as contributing to the potential for additional study.

Chapter 2 (Genz et al., 2008) focuses on the impact of intestinal osmoregulatory mechanisms on whole animal acid-base balance in toadfish acclimated to 9, 35, and 50 ppt salinities. Drinking rate and intestinal water absorption increase in conjunction with increased salinity. As predicted, ambient salinity was observed to strongly influence rectal base excretion and the hypothesis of extra-intestinal compensation was therefore examined by measurements of net extra-intestinal acid fluxes at 9, 35, and 50 ppt

salinities. The most important finding of this chapter was that intestinal base secretion by the intestinal epithelium, increased by ambient exposure to high salinity (and increased drinking), creates an acid-base balance disturbance which is compensated for by increased branchial acid excretion. In general, a whole-animal perspective, employing simultaneous experimentation on integrated osmoregulatory organs, as in Chapter 2, is an uncommon approach to *in vivo* physiological research.

While transporters associated with intestinal osmoregulation and how they are impacted by salinity was the main focus of this dissertation, it is important to note that the primary regulatory factor of osmoregulation in fish is the control of drinking rate, which increases with increasing salinity (Maetz and Skadhauge, 1968; Marshall and Grosell, 2005). Changes in intestinal transport mechanisms following transfer to increased salinity are most relevant when viewed in the context that these changes are not due to salinity *per se*, but rather to changes in the ambient conditions within the gut resulting from changes in the amount of fluid ingested and the composition of this fluid. Increased drinking rate in high salinity continuously provides adequate luminal substrate for Na^+, Cl^-, and water absorption, with capacity for Na^+ absorption exceeding the ingested Na^+, except in fish exposed to highly concentrated seawater (Maetz and Skadhauge, 1968). The fractional absorption of Na^+ and Cl^- by toadfish intestinal epithelia leveled off in high salinity (Fig. 2.7) and fractional water absorption appeared to decrease (Fig. 2.8). The cause of these limitations on tissue ion and water absorption was not immediately apparent, and thus motivated further investigation (Chapter 3).

Chapter 3 quantified salt and water transport by toadfish *in vivo* and by isolated intestinal epithelia to determine the limiting factors for the ability of marine fish to

tolerate elevated salinity. Effective osmoregulation, through transport of water, Na^+, and Cl^- across the intestinal epithelium, was enhanced with decreased ingestion of $MgSO_4$. Thus, the upper salinity limiting survival in toadfish with respect to iono- and osmoregulation was determined by concentration of $MgSO_4$ in the intestinal lumen. In addition to the effects of luminal [$MgSO_4$] on intestinal transport, the minor permeability of these divalent ions (see plasma [Mg^{2+}] in high salinity of normal ionic content versus high salinity water adjusted with NaCl, Table 3.7) was suspected to have impacts on kidney function. Consequently, Chapter 3 examined renal parameters in addition to intestinal transport properties. Ureteral catheterization allowed for *in vivo* sampling of urine and measurement of urine flow rate. Urine flow rate is similar in 35 and 70 ppt seawater-acclimated toadfish, but increased with decreased ambient [$MgSO_4$] (Fig. 3.8). Greater water loss in this treatment indicated the minimal impact of renal processes to osmoregulation with respect to Mg^{2+} and SO_4^{2-} excretion. In addition, enhanced survival of fish with high ambient [NaCl] indicated that branchial ion excretion was not limited in high salinity. Collectively, these three observations indicated that drawback of water from the blood into the intestinal lumen due to luminal [$MgSO_4$], and not branchial or renal factors, was the primary factor determining salinity tolerance in toadfish.

In the two chapters that studied the toadfish (Chaps. 2 and 3), the interaction of the gill with renal processes and intestinal transport was considered on a whole-animal scale. The main requirement of the gill in freshwater is to take up ions, while in seawater the purpose is primarily to excrete ions taken up diffusively or by active intestinal transport. In either scenario, the gill still must also allow for acid-base regulation and excretion of nitrogenous waste. A question raised in Chapter 2 concerns the nature of

branchial acid excretion. Examination of branchial acid transport, particularly with regards to NH_3:NH_4^+ distribution, in the toadfish (a ureotelic species) exposed to high salinity is an interesting area worthy of study in the future.

Toadfish experienced transcriptional changes to ion transporters involved in HCO_3^- transport upon transfer to high salinity. Specifically, the apical Cl^-/HCO_3^- exchanger SLC26a6 (Grosell et al., 2009b) and the basolateral Na^+-HCO_3^- cotransporter 1 were expressed in all intestinal segments, and strongly upregulated in the middle intestine following transfer to 60 ppt seawater. These changes were used as a starting point for a comparative investigation of transporters associated with hypo-osmoregulation in the trout and killifish in Chapter 4 and 5, respectively.

Chapter 4 considers the physiological and transcriptional changes which occurred following transfer from freshwater to 70% seawater. Work on rainbow trout at Bamfield Marine Centre, BC, Canada included measurements of intestinal ion transport using isolated tissue. In addition, quantitative PCR was used to consider the potential post-transfer transcriptional regulation of ion transporters important to both intestinal water uptake and maintenance of acid-base balance. Overall, the *in vitro* experiments suggested that the primary physiological adjustments to increased salinity in rainbow trout occur at 24-72 h following acute transfer from freshwater to 70% seawater.

Surprisingly, intestinal transporters hypothesized to be important to regulation of acid-base balance (SLC26a6, NHE2) were expressed at very low levels in intestinal tissue and did not change following salinity transfer. SLC26a6 has been identified as the intestinal Cl/HCO_3^- exchanger in toadfish (Grosell et al., 2009b) and mefugu (Kurita et al., 2008), but the results of Chapter 4 suggested that SLC26a6 is not the Cl/HCO_3^-

exchanger responsible for intestinal base secretion in trout; thus, the nature of this transporter may vary by species. A role for a basolateral Na^+-dependent H^+ transporter, allowing for maintenance of intracellular acid-base balance following intracellular CA-mediated hydration of CO_2, has been previously described in the toadfish (Grosell and Genz, 2006). Approximately half the HCO_3^- secreted by the toadfish intestine is derived from this process (with the other half attributed to transepithelial transport). In trout, all of the secreted HCO_3^- from the pyloric cecae and anterior intestine can be accounted for by endogenous CO_2 hydration (Grosell et al., 2009a). Increased expression of a basolateral Na^+/H^+ exchanger (NHE1) in response to salinity transfer in the pyloric cecae suggested that apical H^+ transport in the trout intestine occurs not only by V-type H^+-ATPase as previously demonstrated (Grosell et al., 2007) and confirmed in Chapter 4, but also that NHE1 may be important to Na^+ and H^+ transport at the basolateral membrane.

In Chapter 5, the mechanism supporting the large salinity tolerance range of killifish was evident in comparisons of intestinal transport of killifish acclimated to either 35 or 70 ppt seawater. *In vivo* samples of plasma and intestinal fluid confirmed that fish acclimated to hypersalinity had the expected ion transport characteristics for enhanced water absorption, including increased intestinal base secretion (Fig. 5.4). Examination of isolated intestinal tissue identified some of the transport mechanisms important for intestinal osmoregulation in the killifish. Interestingly, it was determined that there was little change to the transport mechanisms following transfer from 35 to 70 ppt seawater. Killifish may have such a large tolerance for salinity change because the mechanisms important for maintenance of water, ion, and acid-base balance under hypersaline conditions are permanent fixtures of the intestinal tissue.

Base secretion by the intestinal epithelia has important consequences for Na^+ and Cl^- absorption, which is critical for the uptake of water, and also for whole-animal acid-base balance (Grosell, 2006). Intestinal base secretion increased in all three fish species upon transfer to increased salinity. Fractional NaCl absorption leveled off in toadfish in 50 ppt seawater, suggesting there are limitations on intestinal water absorption. Chapter 3 identified these limitations as drawback of water due to concentration of luminal $MgSO_4$. Furthermore, the results of Chapters 4 and 5 suggested that increased intestinal base secretion is a function of species-specific salinity tolerance, rather than an absolute effect of ambient salt content.

Intestinal transport is not only influenced by the osmotic pressure differentials impacting fluid movement, but also the impact on both passive and active ion transport, dictated by electrochemical gradients across the intestinal epithelium. The electrophysiology of the isolated intestine of all three fish species was examined. In toadfish intestinal epithelia, TEP was -21.6±2.3 (Grosell and Genz, 2006), which was substantially more negative than the TEP of -8.9±1.0 in FW rainbow trout (Table 4.5) or in killifish acclimated to 35 ppt seawater which was -7.7±0.8 (Fig. 5.5). The negative values in the intestine of all fish species considered in this study were in agreement with the high Cl^-:Na^+ uptake ratio of the intestinal epithelia observed in teleost fish (Grosell, 2006; Wood and Grosell, 2009). Interestingly, at 24 h both killifish transferred to 70 ppt seawater and trout transferred to 70% seawater exhibited an apparently more negative TEP (-8.5±0.8 and -10.0±1.8, respectively), suggesting altered ambient salinity (and thus luminal ionic composition) induced temporary changes to ion transport by the intestinal epithelium.

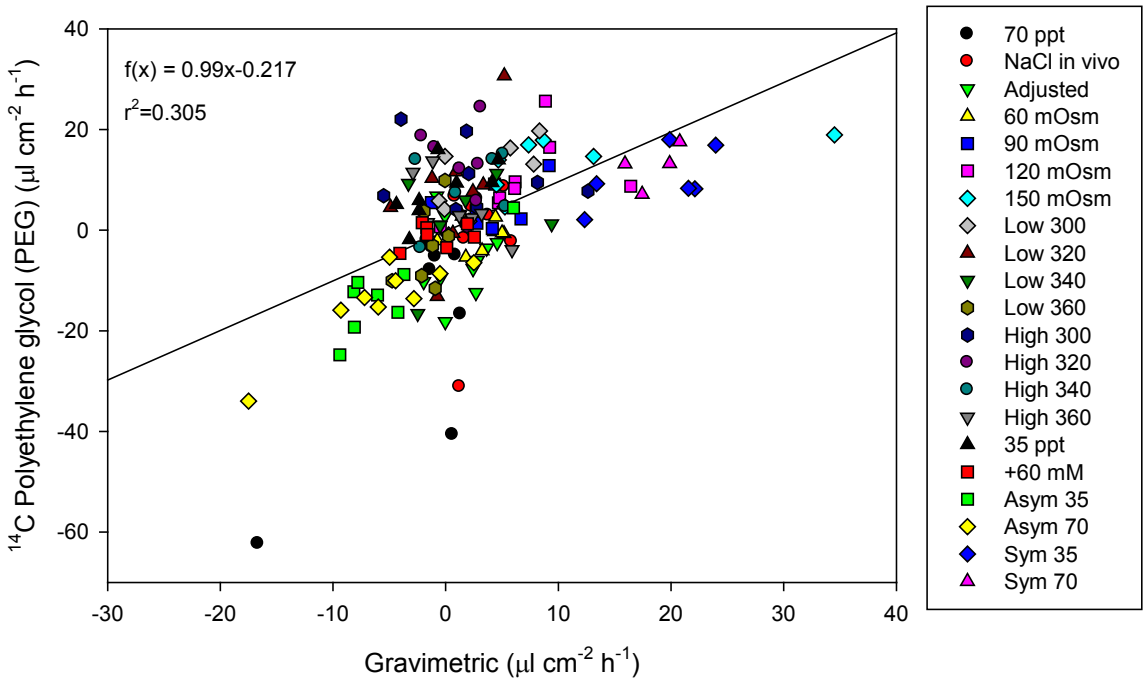
Additionally, during the above research, important information regarding experimental methods commonly used in this field was discovered. Use of isolated tissues for in-depth examination of transport properties is a commonly used method in physiology research. While this has proved to be an effective strategy for investigation of branchial transport in the killifish and intestinal transport in a number of species including trout and toadfish, use of this protocol for the intestine is relatively new. In Chapter 5, the isolated killifish intestinal tract was shown to be unstable under asymmetrical *in vitro* conditions (Figs. 5.1, 5.7-5.8). These observations cast doubt on whether the intestinal epithelium was viable and robust under symmetrical conditions either, and regardless, results from isolated intestine in symmetrical conditions are not physiologically relevant. Thus, while this species seems ideal for the study of plasticity in salinity tolerance, detailed examination by isolation of the intestinal tissue was apparently unfeasible under the conditions tested in this study.

Water flux occurring in the isolated intestinal sacs of toadfish (Chap. 3) and killifish (Chap. 5) was calculated in two ways, by weight (gravimetric) and using ^{14}C polyethylene glycol (PEG) as a tracer. Both methods have arguments for and against their use. Primarily, it is argued that PEG measurements are more accurate, but the protocol requires more steps, and may therefore introduce more experimental errors. Conversely, the gravimetric method is simpler and more accessible (particularly in field conditions, as in Chap. 4), but may sacrifice accuracy. Specifically, as the gravimetric method assesses only differences in weight before and after flux, fluid that is not transported completely across the tissue into the flux chamber, but is no longer in the lumen (i.e. water that is “trapped” in the epithelial cell layer) is included as part of the

post-flux luminal volume. To this end, Fig. 6.1 presents a linear regression of the paired individual water flux measurements (n=4-10) for 21 sets of intestinal sacs calculated using each method. While the various treatments result in a range of flux values, the regression slope of 0.99 indicates that the two methods produce nearly identical results overall. Calculations using PEG measurements were chosen to be presented in both Chaps. 3 and 5 because of the above concerns about the accuracy of the gravimetric method, compared to the PEG method. Not only does this potentially greater accuracy impact the reported water flux, but also the sac volume calculated from these measurements was used in the calculations of base and ion flux.

Experimental techniques ranging from whole-animal to molecular levels allow for a comprehensive understanding of intestinal transport with respect to osmoregulation. Examination of the transport system using isolated intestinal tissue is a valuable method used extensively in the present studies. In addition, the effects of pharmacological agents on the tissue indicates both the type of transport occurring, as well as generalized location of individual transporters (apical or basolateral). Investigation of the distribution of function by tissue segment may be a valuable focus of future research, as the segments of the gastrointestinal tract may be functionally different. Most of the work presented in this dissertation has focused on either the anterior intestine or the entire tract as representative of the overall system. However, the morphological variations of the three species used in the present study highlights the potential for intestinal segment-specific differences in function. The relatively short gastrointestinal tract of the agastric killifish (Babkin and Bowie, 1928) was examined in Chapter 5 both via isolated anterior tissue and as a whole.

Figure 6.1. Linear regression of water flux (µl cm^{-2} h^{-1}) assessed by either the gravimetric method or ^{14}C-PEG activity in intestinal sac preparations



However, the isolated tissue was determined to be unviable in these preparations, and is therefore not an optimal representation of *in vivo* processes. In the trout, the pyloric cecae were investigated in addition to the anterior intestine with regards to gene expression (Chapter 4). (Grosell et al., 2009a). Investigation of gene expression is a good method for identifying the transporters important to salinity acclimation, particularly when *in vivo* and *in vitro* methods are limited, as is the case with *F. heteroclitus*. Additional studies on the intestinal transporters in this species may be a productive area for future study, although proteins associated with osmoregulatory transport are unlikely to vary much following salinity change, as suggested by the limited adjustments to ion and water flux between killifish acclimated to 35 and 70 ppt seawater transfer (Table 5.3, Figs. 5.7, 5.8).

The research discussed above adds to the understanding of ion, water, and acid-base transport as it relates to the variable euryhalinity of teleost fishes. The studies comprising this dissertation contribute to the understanding of both the mechanisms of intestinal osmoregulation and its characteristic parameters most important for salinity tolerance, and add to the literature to date on hypoosmoregulatory mechanisms of teleost fish. While intestinal osmoregulation of fish has been an active research topic for ~80 years (Smith, 1930), studies examining fish in high salinity environments, motivated either due to the natural exposure of the species, or, as is the case in the present work, to "push" the limits of the physiological system, are relatively rare. This dissertation has expanded the knowledge associated with the topic of intestinal mechanisms of hypoosmoregulation by teleost fish in high salinity environments.

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