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

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SUMMARY

Marine teleosts have extracellular fluids less concentrated than their environment, resulting in continual water loss, which is compensated for 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. 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 to 0.70±0.19 ml kg^{-1} h^{-1} in 35 ppt and 1.46±0.22 ml kg^{-1} h^{-1} in 50 ppt. 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, respectively. By contrast, the fraction of imbibed water absorbed decreased from 85.9±3.8% in 9 ppt to 68.8±3.2% in 35 ppt and 61.4±1.0% in 50 ppt. 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, 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 and 403.1±32.9 μmol kg^{-1} h^{-1} at 9, 35 and 50 ppt, respectively), demonstrating a compensatory response to altered intestinal base secretion associated with osmoregulatory demand.

Key words: osmoregulation, HCO_3^- secretion, toadfish, fractional water absorption, drinking rate.

INTRODUCTION

Marine teleosts maintain the osmotic pressure of their extracellular fluids at ~300–350 mosmol l^{-1}, approximately one-third that of seawater (~1000 mosmol l^{-1}) (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 source 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) in exchange for Na^+ (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 CO_2 partial pressure (P_{CO_2}) compared with air-breathing vertebrates (Heisler, 1980; Claiborne, 1997). Hyperventilation to combat acidosis is therefore a relatively inefficient strategy in water-breathing compared with air-breathing animals due to the lower scope for change in P_{CO_2}. 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. The extracellular fluids of the fish are isosmotic to the surrounding water at 9 ppt, reducing the need to drink. Conditions naturally experienced by the gulf toadfish are represented by 35 ppt, while 50 ppt 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.

MATERIALS AND METHODS

Experimental animals

Gulf toadfish (*Opsanus beta* Goode and Bean 1880) were obtained as by-catch from Biscayne Bay, FL, USA by shrimp fishermen and transferred to 62-liter 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 that had continuous flow of aerated, filtered seawater from Biscayne Bay (Bear Cut, 34–37 ppt, 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 were 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 (N=8) was adapted from a previously published method (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 3.7×10^5 Bq l^{-1} [^{14}C]polyethylene glycol-4000 (PEG-4000) (specific activity: 7.585 Bq g^{-1}; NEN-Dupont, Boston, MA, USA) 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 to a final concentration of 0.2 g 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 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, USA). 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, mass of individual fish, including the mass 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 h 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-liter aquarium with filtered seawater (~35 ppt) 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, USA) dissolved in 35 ppt seawater to give a final salinity of 60 ppt. Fish were acutely transferred to 60 ppt, 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 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 10 000 g) (Eppendorf 5415D, Hamburg, Germany). Following anesthesia in 0.2 g 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 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 represent a lower and higher salinity tolerance 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 (in mmol l^{-1}): 112, 133, 12 and 5.3, respectively, for the 9 ppt treatment; 454, 440, 46 and 24, respectively, for the 35 ppt treatment and 797, 649, 87 and 32 mmol l^{-1}, 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 µmol l^{-1} in all cases.

Cannulation of acclimated toadfish

Following acclimation to 9, 35 and 50 ppt, toadfish were exposed to 0.2 g l^{-1} MS-222 in the same salinity water to which they were acclimated until anesthetized, and gills were perfused with 0.1 g l^{-1} MS-222 in the appropriate salinity throughout surgery. A caudal

incision allowed for insertion of a catheter of polyethylene tubing (PE50) (Intramedic, Becton Dickinson & Co., Sparks, MD, USA) into the caudal artery or vein. The catheter was enclosed in a short sleeve of larger tubing (PE160), 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 liter) 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 in pre-weighed 15 ml Falcon tubes as described above for the acutely transferred fish.

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 mass. 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 mol l^{-1} HCl and then titrated back to the initial pH using 0.02 mol l^{-1} NaOH. For some precipitate samples, 0.2 mol l^{-1} HCl and 0.02 mol l^{-1} 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 (GS-1200; Gilmont Instruments, Barrington, IL, USA). In addition to analyzing ionic composition of the intestinal and rectal fluids, 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, USA; coupled to a Radiometer PHM201 pH meter), and for total CO_2 using a total CO_2 analyzer (Corning 965, Medfield, MA, USA). Anion concentrations from all fluid samples were quantified by anion chromatography (Dionex 120, Sunnyvale, CA, USA), while cations were analyzed by fast sequential flame atomic absorption spectrometry (Varian 220, Palo Alto, CA, USA) using an air/acetylene flame. Ammonium content of water samples was determined by colorimetric assay (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 Eqn 1 while the CO_3^{2-} concentration was determined from Eqn 2, in both cases using a pK_{II} of 9.46 for toadfish gut fluids (Wilson et al., 2002):

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

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

Note that this approach ignores the contribution from molecular CO_2, which is justified by molecular CO_2 concentrations of less than 10^{-9} mol l^{-1} 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 $2\times[CO_3^{2-}]$. 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 subsequent 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 h experimental period.

Data presentation and statistical analysis

All values are given as means ± s.e.m. The control (0 h post-transfer in acutely transferred toadfish or 35 ppt in acclimated toadfish) and experimental values were compared using Student's *t*-tests with Bonferroni multi-sample comparison correction. Data sets not found to be normally distributed were compared using Mann–Whitney rank sum test. Differences between means were considered statistically significant when P<0.05.

RESULTS

Drinking rate and rectal excretion

In this study, the drinking rate of *O. beta* in 50 ppt 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, 0.70±0.19 ml kg^{-1} h^{-1} in 35 ppt and 1.46±0.22 ml kg^{-1} h^{-1} in 50 ppt (data not shown).

Acute transfer to hypersalinity

Toadfish acutely transferred from 35 to 60 ppt displayed a transient disturbance of acid–base balance. Total CO_2 of both blood plasma and intestinal fluids transiently increased immediately after salinity transfer, followed by a significant reduction in plasma total CO_2 at 96 h post-transfer (Fig. 1). In the intestinal fluid, Cl^- concentration

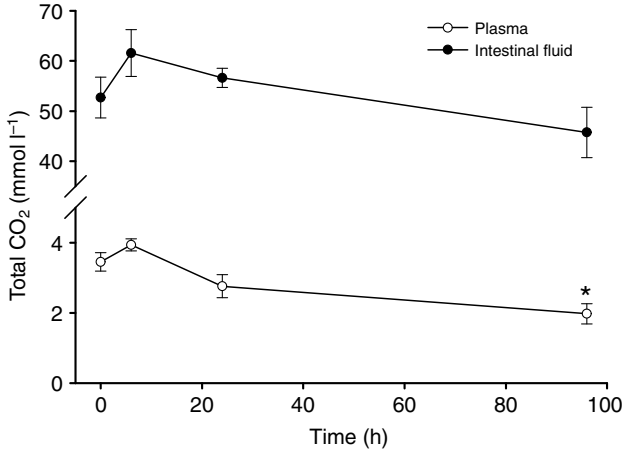

Fig. 1. Total CO_2 in the blood plasma (open circles) and intestinal fluid (filled circles) of toadfish acutely transferred from 35 to 60 ppt and sampled at 0, 6, 24 and 96 h post-transfer. Values are means ± s.e.m. ($N \geqslant 7$). An asterisk (*) indicates statistically significant difference from 0 h value ($P<0.05$).

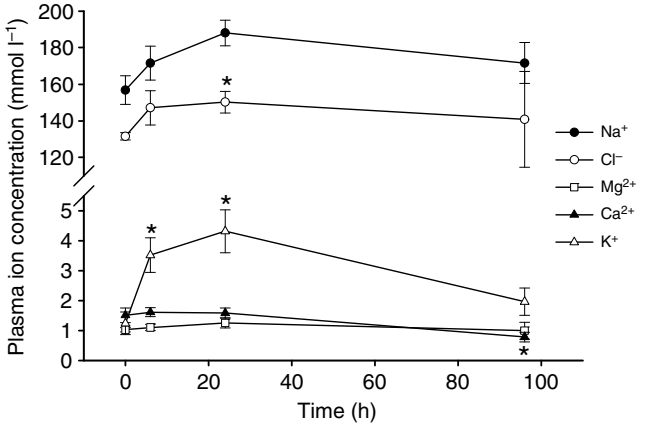

Fig. 3. Concentrations of Na^+, Cl^-, Mg^{2+}, Ca^{2+} and K^+ in blood plasma of toadfish acutely transferred from 35 to 60 ppt and sampled at 0, 6, 24 and 96 h post-transfer. Values are means ± s.e.m. ($N \geqslant 6$). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

was elevated at both 24 and 96 h post-transfer (Fig. 2), which coincides with the decrease to initial values seen in total CO_2 in the intestinal fluid at these time points (Fig. 1). By contrast, Na^+, Ca^{2+}, K^+ and SO_4^{2-} concentrations in the intestinal fluids were relatively stable over time. However, in the plasma of toadfish transferred acutely to 60 ppt (Fig. 3), both Na^+ and Cl^- increased during the first 24 h 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. 3) following transfer to 60 ppt.

Gradual acclimation to low and high salinities

Plasma HCO_3^- equivalents in toadfish acclimated to both 9 ppt and 50 ppt were not significantly different from those of samples obtained from toadfish in 35 ppt. Bicarbonate equivalents in the rectal fluid are relatively constant in all salinities (Fig. 4). Rectal fluid pH was alkaline in all samples but was significantly decreased in 50 ppt samples and increased in isosmotic conditions (9 ppt) compared with control values (35 ppt). Similarly, plasma pH was lower at 50 ppt and higher at 9 ppt compared with 35 ppt. 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.

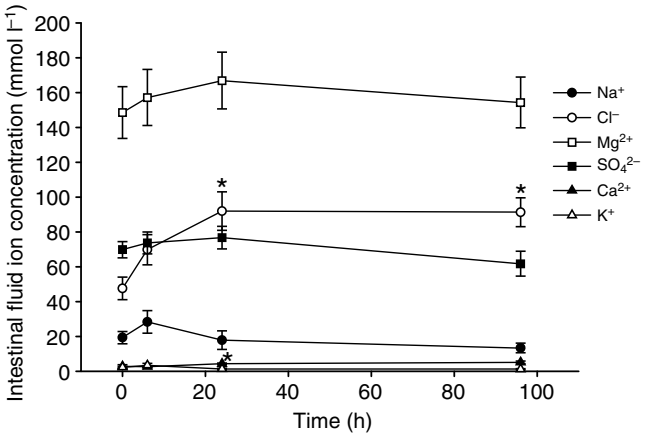

Fig. 2. Concentrations of Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, Ca^{2+} and K^+ in intestinal fluids of toadfish acutely transferred from 35 to 60 ppt and sampled at 0, 6, 24 and 96 h post-transfer. Values are means ± s.e.m. ($N \geqslant 5$). An asterisk (*) indicates statistically significant difference from 0 h value ($P<0.05$).

All measured ions in the plasma (Na^+, Cl^-, Mg^{2+}, Ca^{2+}, K^+) tended to be higher in 50 ppt than in 35 ppt (Table 1), although none of these differences was statistically significant. In the rectal fluid of acclimated toadfish (Table 2), the concentrations of K^+ and Na^+ decreased from 9 to 35 ppt whereas SO_4^{2-} increased. Similarly, Mg^{2+} greatly increased both from 9 to 35 ppt, and from 35 to 50 ppt. The absolute ion excretion rate ($\mu mol\,kg^{-1}\,h^{-1}$) from the rectum over 48 h is shown in Fig. 5. In toadfish acclimated to 50 ppt, Mg^{2+} and Cl^- excretion was significantly greater than in 35 ppt, with a similar, but not statistically significant, trend for SO_4^{2-}, Na^+, K^+ and Ca^{2+}. Intestinal excretion of HCO_3^-, Ca^{2+} and Mg^{2+} occurs 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. 5). The fraction of Ca^{2+} excreted as precipitate increased from 26.8% in 35 ppt to 61.2% in 50 ppt fish, while the percentage of excreted Mg^{2+} in the precipitate was very low (2.7% in 35 ppt and 3.6% in 50 ppt). 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 to 23.4% in 50 ppt fish (Fig. 6). Extra-intestinal net acid excretion (sum of titratable acid flux and total ammonia flux) also increased with increasing salinity (Fig. 6). Interestingly, total ammonia excretion in itself tended to increase with increasing salinity ($P<0.062$).

DISCUSSION

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

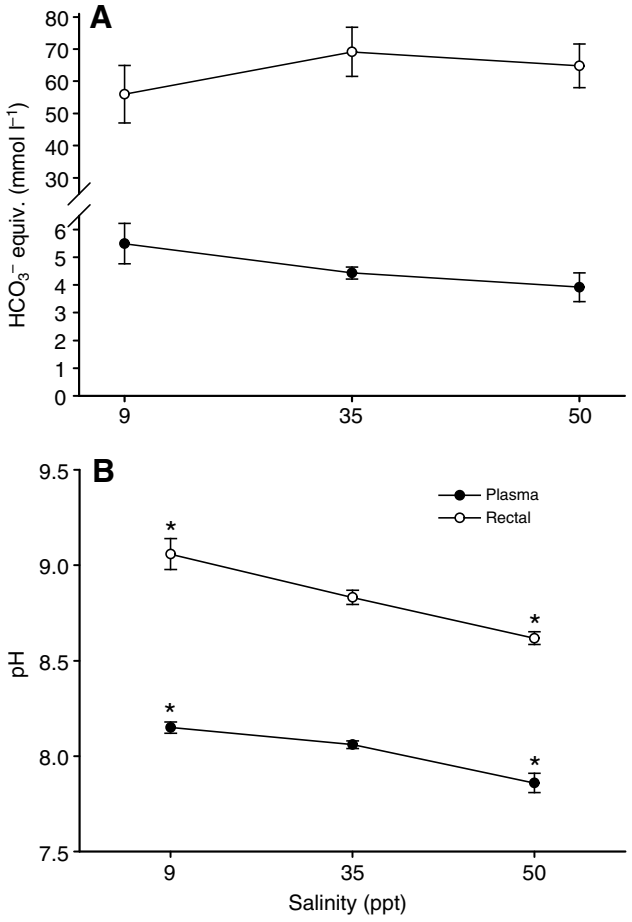

Fig. 4. (A) HCO_3^- equivalents and (B) pH of blood plasma and rectal fluids collected at 48 h from toadfish acclimated to 9, 35 and 50 ppt. Values are means ± s.e.m. ($N \geq 5$). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

plasma ion composition between 5 and 50 ppt but not in 70 and 2.5 ppt (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 for at least 96 h. However, a preliminary experiment in which fish were acclimated to 60 ppt and underwent cannulation surgery resulted in high mortality. The acclimation salinity was therefore decreased to 50 ppt 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 isosmotic salinities have little need for water absorption across the gastrointestinal tract. Thus, as expected, the drinking rate of toadfish in isosmotic conditions is low, demonstrated by the low rate of fluid excretion from the rectum at 9 ppt compared with secretion rates from fish in 35 and 50 ppt. 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 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, 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), 2.63±0.97 (35 ppt) and 3.89±0.57 ml kg^{-1} h^{-1} (50 ppt). Considering these observations of Mg^{2+} and SO_4^{2-} combined, drinking rates can be estimated to be 1.38±0.30, 2.60±0.92 and 3.82±0.58 ml kg^{-1} h^{-1} in 9, 35 and 50 ppt, 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 were significantly higher than at 9 ppt 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 is not significantly different from that directly measured in non-cannulated toadfish acclimated to 50 ppt (3.82±0.58 ml kg^{-1} h^{-1} compared with 3.24±0.34 ml kg^{-1} h^{-1}, respectively). The measured rate at 50 ppt is higher than the mean rate estimated for cannulated fish in 35 ppt (2.60±0.92 ml kg^{-1} h^{-1}) and higher than those previously reported for toadfish acclimated to 30 ppt (Grosell et al., 2004). Admittedly, if $MgSO_4$ was absorbed by the intestine, our calculations

Table 1. Blood plasma ion concentrations of *O. beta* acclimated to 9, 35 and 50 ppt

	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

Concentrations (mmol l^{-1}) of Na^+, Cl^-, Mg^{2+}, Ca^{2+} and K^+ in blood plasma of toadfish acclimated to 9, 35 and 50 ppt for a minimum of 14 days. Values are means ± s.e.m. ($N \geq 4$).

Table 2. Rectal fluid ion concentrations of *O. beta* acclimated to 9, 35 and 50 ppt

	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

Concentrations (mmol l^{-1}) 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. Values are means ± s.e.m. ($N \geq 4$). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

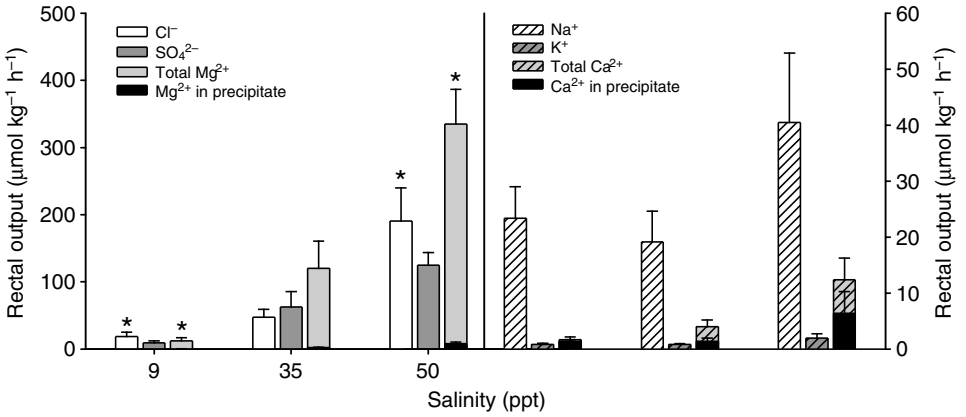

Fig. 5. Mg^{2+}, Cl^-, SO_4^{2-}, Na^+, Ca^{2+} and K^+ excreted in rectal fluid over 48 h by toadfish acclimated to 9, 35 and 50 ppt ($\mu mol\,kg^{-1}\,h^{-1}$). The fraction of total Mg^{2+} and Ca^{2+} excreted in precipitates is shown in stacked bars. Values are means ± s.e.m. ($N \geq 4$). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

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 50 ppt fish 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, 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. 7). In fish acclimated to 35 ppt, 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 includes 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 (1.21±0.27 ml $kg^{-1}\,h^{-1}$) and 50 ppt (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). 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 Fig. 8, which displays 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, apparently because fractional water absorption at 35 ppt 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, 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 to 60 ppt 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 statistically elevated from the initial (control) value, which is likely to be 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 is most likely to be the result of a constant seawater ingestion rate balanced by a constant Cl^- absorption rate, established during this period. 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 that 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, 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, respectively). Consistent with this observation, plasma pH is significantly lower in toadfish acclimated to 50 ppt than in 35 ppt-acclimated fish, 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

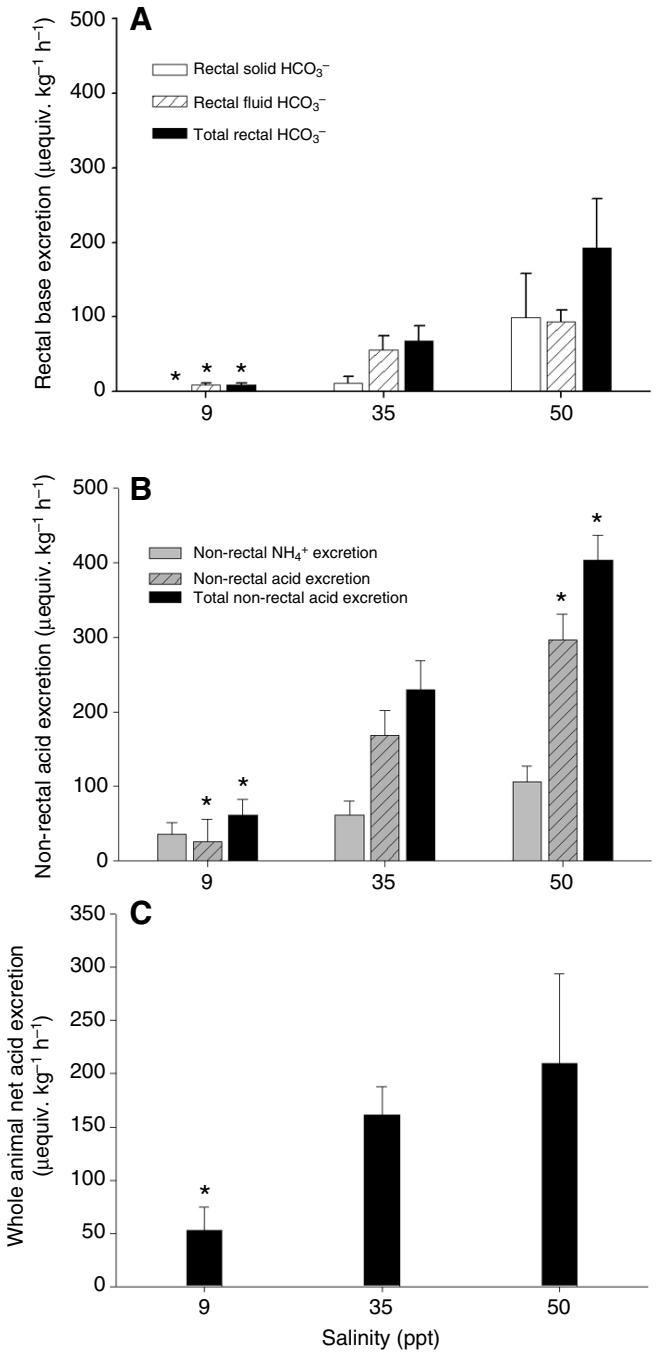

Fig. 6. Rectal and non-rectal excretion of acid–base equivalents over 48 h (μequiv kg^{-1} h^{-1}) by toadfish acclimated to 9, 35 and 50 ppt and fitted with rectal collection sacs. (A) Total rectal base excretion is the sum of base equivalents in both rectal fluid and precipitate. (B) Total non-rectal 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. ($N \geq 6$). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

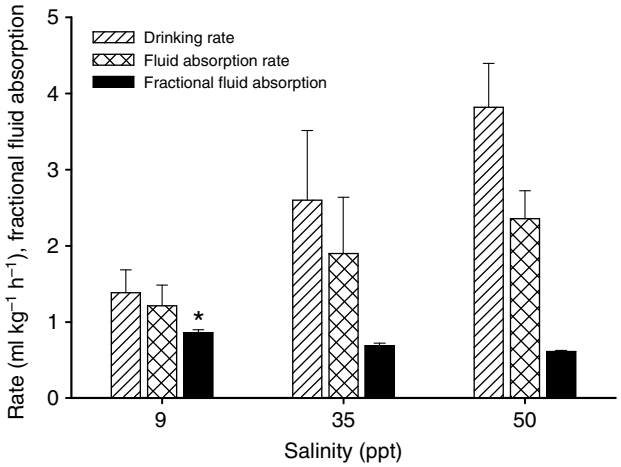

Fig. 7. Estimated drinking rate (ml kg^{-1} h^{-1}), fluid absorption rate (ml kg^{-1} h^{-1}) and fractional fluid absorption of toadfish acclimated to 9, 35 and 50 ppt. Drinking rates were estimated based on Mg^{2+} and SO_4^{2-} concentrations in rectal fluid samples and 9, 35 and 50 ppt water. Values are means ± s.e.m. (N=6). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

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 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 (9.3±2.7 μmol kg^{-1} h^{-1}) and 50 ppt (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. 6C). 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 was analyzed immediately for pH in a custom-made gastight, water-jacketed chamber fitted with a Radiometer combination pH electrode (4000-8). Subsequently, a fraction of these individual blood samples was 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 accounts, at least in part, for the relatively high pH values obtained in the present study. However, 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

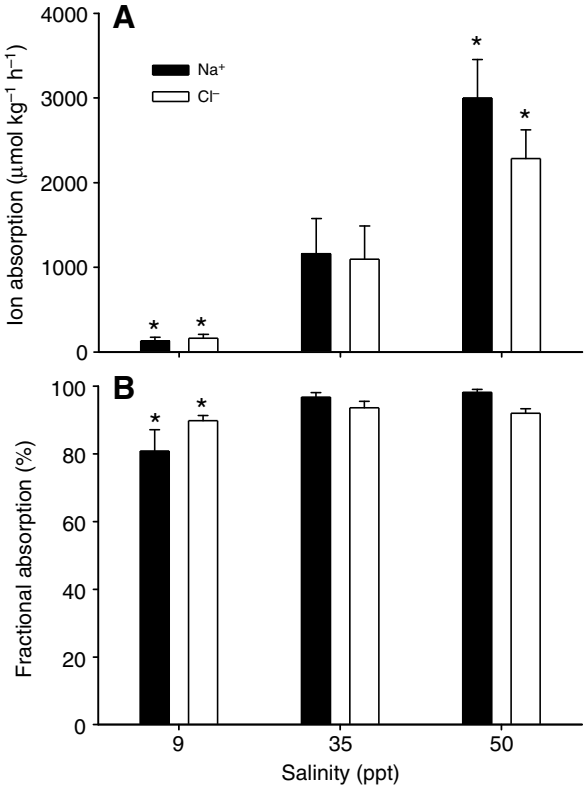

Fig. 8. Calculated (A) absorption rate (μmol kg^{-1} h^{-1}) and (B) fractional absorption of ingested Na^+ and Cl^- by toadfish acclimated to 9, 35 and 50 ppt based on Na^+ and Cl^- concentrations in rectal fluid samples and 9, 35 and 50 ppt water. Values are means ± s.e.m. ($N \geqslant 5$). An asterisk (*) indicates statistically significant difference from 35 ppt ($P<0.05$).

present study demonstrate an increase in extra-intestinal, presumably branchial, net acid excretion in elevated salinity (Fig. 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, are clearly 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 adequate excretion of nitrogenous waste and would contribute to the correction of acid–base balance. Furthermore, it might act as a mechanism for energy retention, 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 displays a blood-side negative TEP in seawater (Evans, 1980). In contrast to NH_4^+, excretion of NH_3 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 with 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.

Conclusions 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 1, Fig. 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 performing integrative experiments to clarify how the renal processes might be related to the salinity response observed in the present 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 that 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 that 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 would likely 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).

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