The Journal of Experimental Biology 213, 2681-2692
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doi:10.1242/jeb.039164

Acid–base responses to feeding and intestinal Cl^- uptake in freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus*, an agastric euryhaline teleost

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Accepted 26 April 2010

SUMMARY

Marine teleosts generally secrete basic equivalents (HCO_3^-) and take up Na^+ and Cl^- in the intestine so as to promote absorption of H_2O. However, neither the integration of these functions with feeding nor the potential role of the gut in ionoregulation and acid–base balance in freshwater have been well studied. The euryhaline killifish (*Fundulus heteroclitus*) is unusual in lacking both an acid-secreting stomach and a mechanism for Cl^- uptake at the gills in freshwater. Responses to a satiation meal were evaluated in both freshwater- and seawater-acclimated killifish. In intact animals, there was no change in acid or base flux to the external water after the meal, in accord with the absence of any post-prandial alkaline tide in the blood. Indeed, freshwater animals exhibited a post-prandial metabolic acidosis ('acidic tide'), whereas seawater animals showed no change in blood acid–base status. *In vitro* gut sac experiments revealed a substantially higher rate of Cl^- absorption by the intestine in freshwater killifish, which was greatest at 1–3 h after feeding. The Cl^- concentration of the absorbate was higher in preparations from freshwater animals than from seawater killifish and increased with fasting. Surprisingly, net basic equivalent secretion rates were also much higher in preparations from freshwater animals, in accord with the 'acidic tide'; in seawater preparations, they were lowest after feeding and increased with fasting. Bafilomycin (1 μmol l^{-1}) promoted an 80% increase in net base secretion rates, as well as in Cl^- and fluid absorption, at 1–3 h post-feeding in seawater preparations only, explaining the difference between freshwater and seawater fish. Preparations from seawater animals at 1–3 h post-feeding also acidified the mucosal saline, and this effect was associated with a marked rise in P_{CO_2}, which was attenuated by bafilomycin. Measurements of chyme pH from intact animals confirmed that intestinal fluid (chyme) pH and basic equivalent concentration were lowest after feeding in seawater killifish, whereas P_{CO_2} was greatly elevated (80–95 Torr) in chyme from both seawater and freshwater animals but declined to lower levels (13 Torr) after 1–2 weeks fasting. There were no differences in pH, P_{CO_2} or the concentrations of basic equivalents in intestinal fluid from seawater versus freshwater animals at 12–24 h or 1–2 weeks post-feeding. The results are interpreted in terms of the absence of gastric HCl secretion, the limitations of the gills for acid–base balance and Cl^- transport, and therefore the need for intestinal Cl^- uptake in freshwater killifish, and the potential for O_2 release from the mucosal blood flow by the high P_{CO_2} in the intestinal fluids. At least in seawater killifish, H^+-ATPase running in parallel to HCO_3^-:Cl^- exchange in the apical membranes of teleost enterocytes might reduce net base secretion and explain the high P_{CO_2} in the chyme after feeding.

Key words: alkaline tide, chloride uptake, bicarbonate secretion, chyme P_{CO_2}, intestine.

INTRODUCTION

Recent studies have demonstrated that at least two species of fish, the marine spiny dogfish shark (Wood et al., 2005; Wood et al., 2007b) and the euryhaline rainbow trout, in both freshwater (Bucking and Wood, 2008; Cooper and Wilson, 2008) and seawater (Bucking et al., 2009) exhibit the presence of a 'post-prandial alkaline tide'. In this respect, they are similar to many higher vertebrates, where a systemic metabolic alkalosis is commonly seen following a meal (reviewed by Brunton, 1933; Wang et al., 2001; Niv and Fraser, 2002). The cause of the alkalosis in higher vertebrates is activation of metabolic acid secretion (as HCl) by the cells of the stomach: equimolar transport of HCO_3^- ions (metabolic base) into the extracellular fluid results in alkalinization of the blood. A similar explanation likely applies in fish because intra-gastric administration of the H^+ pump inhibitor omeprazole attenuated the alkaline tide in the dogfish shark (Wood et al., 2009), just as it does in higher vertebrates such as toads (Andersen and Wang, 2003), snakes (Andrade et al., 2004) and humans (Sachs et al., 1995; Niv and Fraser, 2002).

In higher vertebrates, compensation for the alkaline tide occurs in the short term by restricting ventilation so as to allow blood P_{CO_2} levels to rise (i.e. compensatory respiratory acidosis), and in the longer term by secreting HCO_3^- from pancreatic, biliary and duodenal glands into the small intestine, thereby neutralizing the chyme in preparation for its enzymatic digestion (Wang et al., 2001). A small component might also be excreted in the urine (Bence-Jones, 1845; Roberts, 1859; Brunton, 1933; Rune, 1966; Vaziri et al., 1980). However, in water-breathers, the gills are hyperventilated with respect to CO_2 excretion, and therefore fish do not have the luxury of reducing ventilation because O_2 uptake would be compromised (reviewed by Gilmour, 2001). Instead, at least in the dogfish shark, a massive excretion of metabolic base across the gills occurs for many hours after feeding, thereby limiting the magnitude of systemic metabolic alkalosis, and avoiding a possibly fatal rise in blood pH (Tresguerres et al., 2007; Wood et al., 2007a; Wood et al., 2009). A very similar response of comparable magnitude is seen in the rainbow trout in freshwater (Bucking and Wood, 2008), but curiously exactly the opposite occurs in the rainbow trout in

seawater, where a sustained uptake of metabolic base (equivalent to metabolic acid secretion) is seen after a meal (Bucking et al., 2009). Bucking et al. (Bucking et al., 2009) proposed that this occurred to satisfy elevated rates of net base secretion by the intestine in feeding fish. Intestinal secretion of basic equivalents is linked at least in part to Cl^- uptake, and occurs largely in the form of HCO_3^-. It plays a key role in the osmoregulatory strategy of marine teleosts by precipitating Ca^{2+} and Mg^{2+} from intestinal fluids and thereby promoting fluid absorption (reviewed by Wilson et al., 2002; Grosell, 2006). Taylor and Grosell (Taylor and Grosell, 2006) provided evidence that intestinal base secretion by means of Cl^-/HCO_3^- exchange was greatly enhanced in the marine gulf toadfish post feeding and have since demonstrated that it occurs to an extent sufficient to account for a lack of an alkaline tide in this species (Taylor and Grosell, 2009). Furthermore, Bucking et al. (Bucking et al., 2009) used an *in vitro* gut sac technique to demonstrate that intestinal HCO_3^- secretion and Cl^- uptake rates were greatly elevated after feeding in seawater-acclimated trout but remained unchanged after feeding in freshwater-acclimated trout. While information is limited, the few available *in vivo* studies indicate that intestinal HCO_3^- concentrations are low in freshwater teleosts, suggesting that secretion rates are also low (Wilson, 1999; Grosell, 2006).

With this background in mind, we investigated the impact of feeding on acid–base balance (by measurements of whole-animal acid–base flux rates and blood acid–base status) and intestinal function (by *in vitro* gut sac experiments) in the euryhaline common killifish, *Fundulus heteroclitus*, in both freshwater and seawater. For several reasons, we suspected that the killifish would respond very differently from the euryhaline rainbow trout, and thereby cast light on the linkages between feeding, acid–base balance and osmoregulatory strategies. First, the killifish lacks a stomach and indeed appears to have an entirely alkaline digestive system (Babkin and Bowie, 1928). Second, an extensive literature has demonstrated that, in seawater, the killifish behaves physiologically like most other euryhaline and stenohaline teleosts that have been studied, but in freshwater its ionoregulatory mechanisms are unusual (Péqueux et al., 1988; Wood and Marshall, 1994; Marshall, 1995; Marshall and Bryson, 1998; Marshall and Singer, 2002; Marshall, 2003; Wood and Laurent, 2003). These differences in freshwater include a virtual absence of active Cl^- uptake at the gills (Patrick et al., 1997; Wood and Laurent, 2003) and, perhaps linked to this, an inability to manipulate active branchial uptake rates of Na^+ and Cl^- in response to internal acid–base disturbances – i.e. an apparent lack of direct Na^+ influx/acid efflux and Cl^- influx/base efflux linkage mechanisms (Patrick et al., 1997; Patrick and Wood, 1999). Instead, excretion of acidic and basic equivalents seems to occur through differential modulation of Na^+ and Cl^- efflux rates at the gills. The absence of Cl^- uptake at the gills suggests that the diet must become the key route of Cl^- uptake when the fish are living in freshwater. While this has never been directly proven, Scott and colleagues (Scott et al., 2006) used *in vitro* gut sac trials to show that Cl^- uptake (also Na^+ uptake) by the intestinal tract is upregulated during acclimation to freshwater.

Our specific hypotheses were that: (1) in intact animals, neither freshwater- nor seawater-acclimated killifish would exhibit an elevated post-prandial base flux to the external water or an alkaline tide in the blood because there would be no elevation of HCl secretion by the tract; (2) in isolated gut sacs, base secretion would be greater in seawater-acclimated killifish and would be upregulated to a greater extent after feeding than in freshwater-acclimated killifish; (3) in these same *in vitro* preparations, Cl^- uptake would be greater in freshwater-acclimated killifish and would be upregulated to a greater extent after feeding so as to acquire this valuable ion from the food; and (4) these differences would be reflected in measurements of the acid–base composition of intestinal chyme taken from intact animals at various times after feeding. Additionally, unexpected differences between freshwater and seawater fish after feeding prompted an *in vitro* examination of the effects of the V-type ATPase inhibitor bafilomycin at this time.

MATERIALS AND METHODS

Experimental animals

Common killifish of the northern subspecies (*Fundulus heteroclitus macrolepidotus* L.; 2–6 g) were purchased from Aquatic Research Organisms (Hampton, NH, USA) who obtained them by beach-seining of local tidal flats. At the University of Miami (Fl, USA), they were held at a density of 30–50 animals per 50 l tank for at least two months before experiments. For freshwater acclimation, salinity was changed gradually over 48 h in several of the tanks, and then the animals were held in flowing freshwater for at least 20 days before test. The composition of Miami seawater (37.5 ppt) and freshwater have been reported by Wood and Grosell (Wood and Grosell, 2008). For simplicity, freshwater-acclimated and seawater-acclimated animals are termed freshwater killifish and seawater killifish, respectively, throughout the remainder of this paper. Acclimation and experimental temperature was 22–24°C. In both fresh water and sea water, the animals were fed Aqua Max sinking pellets (50% protein) with the following measured ionic composition: Na^+ 230, Cl^- 170, Ca^{2+} 628, Mg^{2+} 77 and K^+ 326 $\mu mol\,g^{-1}$, at a daily ration of approximately 1.5%. *In vivo* experiments were performed in the spring of 2008, *in vitro* gut sac experiments in the spring of 2009, chyme sampling experiments in the summer of 2009, and blood sampling and bafilomycin experiments in the spring of 2010. The standard protocol was to keep the fish on the 1.5% daily ration before an experiment, then fast them for 48 h, followed by administration of a satiation meal. Test dissections showed that the ingested ration was typically 3–4% of body mass.

In vivo experiments

In order to make flux measurements on individual animals before and after feeding, it was necessary to isolate the fish in separate containers and train them to feed under these conditions. Each container was fitted with its own supply of fresh water (N=11) or sea water (N=12) supply, as appropriate, and its own airline. The containers were oblong polyethylene food storage containers (volume 500 ml) covered in black plastic at the rear (for shelter) but not shielded at the front, where a small feeding flap was cut in the lid. The fish were trained to feed by dropping individual food pellets by hand through the feeding flap at 10:00–11:00 a.m. each day. The process took 4–5 days, by which time most fish were consuming their regular 1.5% ration. Fish that would not learn to feed were not used. Daily feeding was stopped 36 h before the start of an experiment to ensure gut clearance and a good appetite for the experimental feeding event. Time for complete gut clearance of the last meal in killifish is <24 h (C.M.W., unpublished observations).

At the start of an experiment, water flow to the container was stopped while aeration was maintained, thereby ensuring mixing, and the water level set to a known precise volume (usually 400 ml). The control pre-feeding flux period (12 h) was started at 22:00 by drawing duplicate 15 ml water samples and ended at 10:00 the next day by drawing duplicate samples again. The water flow was then

restarted to thoroughly flush the chambers. The fish were fed by hand to satiation at this time (i.e. approximately 48 h after their last meal), a process that took about 15 min, the containers inspected to make sure no uneaten food remained and then the water flow was suspended and another flux period started at 11:00. Duplicate water samples were taken at 11:00, 15:00, 19:00 and 23:00. The chambers were then flushed again, and a final overnight flux period started, which ended at 11:00 the next day. Water samples were analyzed for titratable alkalinity and total ammonia concentration, as outlined below.

Blood sampling experiments

In view of the small size of the fish, it was not possible to measure blood acid–base status by cannulation. Therefore, we adopted the technique of Patrick and colleagues (Patrick et al., 1997) in which blood is sampled by terminal caudal puncture and tonometered to a fixed P_{CO_2} to remove any 'respiratory' disturbance (i.e. P_{CO_2} elevation) associated with sampling. The remaining 'metabolic' acid–base status can then be determined by measuring pH and plasma HCO_3^-.

Killifish were kept in large groups (30–50 animals per 50 l tank) in either fresh water or sea water and fed a 1.5% ration daily. As in the *in vivo* experiments, feeding was suspended for 48 h, and then the fish were fed to satiation. Blood samples were taken under control conditions (after a fast of 48 h) and at 1, 3, 7 and 18 h post-feeding. At the time of sampling, killifish (*N*=7–9 at each point) were quickly anaesthetized in MS-222 (0.2 g l^{-1}) neutralized with NaOH, and a blood sample (typically 30–40 μl) was quickly drawn into a heparinized gas-tight Hamilton 100 μl-syringe by caudal puncture. The needle had been cut off and beveled so as to sample at the correct depth. The fish survived the procedure, but each fish was sampled only once.

Each sample was then equilibrated, using a precision gas mixture (0.3% CO_2 and 99.7% O_2), to the same P_{CO_2} of 2.25 Torr, which was chosen as a typical *in vivo* P_{CO_2} for teleost fish. The tonometer was the top half (rounded) of a cut-off 0.2-ml bullet tube, mounted upside-down inside a 1.5-ml centrifuge tube. The latter was fitted with inflow and outflow ports to carry the gas in and out, and was mounted in a vigorous shaker in a water bath at 23°C. The gas mixture passed through two humidifying flasks and a water trap before reaching the tonometers. Tests showed that 20 min equilibration was adequate. An Accumet 13-620-96 combination glass microelectrode (Fisher Scientific) fitted snugly into the tonometer flasks and was used to measure pH; the samples were then centrifuged (5000 ***g*** for 2 min) inside their 1.5-ml tube holders, and plasma decanted (back into a Hamilton syringe) for measurement of total CO_2 using a Corning 965 total CO_2 analyzer.

In vitro gut sac experiments

Killifish were kept in large groups in fresh water or sea water, as in the blood-sampling experiments, and fed a 1.5% ration daily. Again, feeding was suspended for 48 h, and then the fish were fed to satiation. Fish were sacrificed for gut sac experiments at 1–3 h post-feeding, 12–24 h post-feeding and after 7–14 days of fasting (*N*=19–27 at each time). A modified Cortland saline (Wolf, 1963) was used for all gut sac preparations and was placed on both mucosal and serosal surfaces (i.e. symmetrical conditions). The composition, in mmol l^{-1}, was NaCl 144, KCl 5.1, $CaCl_2.2H_2O$ 1.4, $MgSO_4.7H_2O$ 1.9, $NaHCO_3$ 11.9, Na_2HPO_4 2.9, glucose 5.5. The saline was gassed with a 0.3% CO_2 and 99.7% O_2 mixture (i.e. nominal P_{CO_2}=2.25 Torr, P_{O_2}>700 Torr) and the pH was then adjusted to 8.12 with small amounts of 1 mol l^{-1} HCl. In some of these experiments, trace amounts of radiolabelled polyethylene glycol of various molecular masses (400, 900, 4000 Da) were present in the mucosal saline for the purposes of another study evaluating the paracellular permeability of the gut epithelium; analysis of the data indicated that these had no effect on any of the parameters measured in the present study.

Methods generally followed those developed earlier by Scott and colleagues (Scott et al., 2006) for *in vitro* gut sac preparations of *Fundulus heteroclitus.* Fish were lightly anaesthetized in MS-222 (0.1 g l^{-1}), killed by a blow to the head, and the intestinal tract dissected out while the fish was kept cold on ice. The killifish lacks a stomach, with the oesophagus entering into the anterior intestine through a pylorus-like sphincter; anterior, mid and posterior sections can be identified, corresponding to the first, second and third portions in Fig. 1 of Babkin and Bowie (Babkin and Bowie, 1928). These were made into a single sac preparation. The distal 10% of the tract was found to be very fragile (at the point where the posterior intestine joins the rectum) and therefore was excluded from the preparation to prevent leakage. Heat-flared PE-50 polyethylene tubing was inserted, and the tract was flushed thoroughly to clear out food and

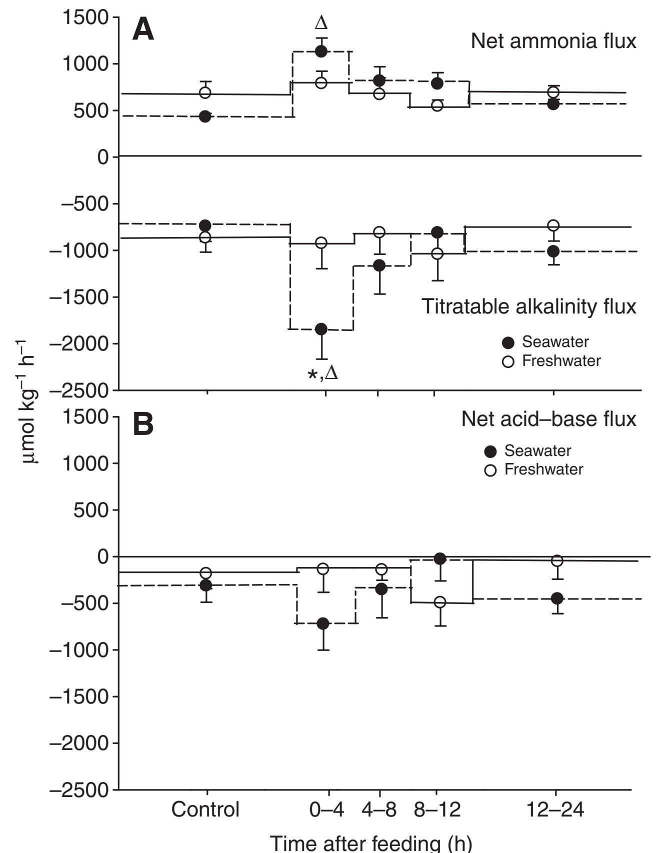

Fig. 1. Changes in (A) net ammonia flux (J_{Amm}) and titratable alkalinity flux (J_{TAlk}) and (B) net acid–base flux ($J_{net H^+}$) to the external water following a satiation meal in *Fundulus heteroclitus* acclimated to either seawater (solid circles; *N*=12) or freshwater (open circles; *N*=11). Means ± 1 s.e.m. Triangles indicate means significantly different (*P*<0.05) from the pre-feeding value within a treatment group, and asterisks indicate means significantly different between treatment groups at a particular time. In (B), a net basic equivalent flux to the environment is shown by a negative value. There were no significant changes in $J_{net H^+}$ at any time after feeding in either seawater or freshwater killifish, and overall $J_{net H^+}$ remained not significantly different from zero.

chyme, using ice-cold saline. One end of the intestine was then tied off, and the catheter secured into the other end with a silk suture. The sac was then filled with saline (typically 0.2–0.6 ml, depending on fish size), taking care not to trap air bubbles and an initial transepithelial potential (TEP) measurement was made (as described below). The catheter was then sealed with a pin. A sample of the filling saline was taken for analysis. The preparation was blotted on tissue paper in a standardized manner, weighed to 0.1 mg accuracy on an analytical balance, then suspended in a Falcon tube containing 6 ml of the same saline. The external saline was continually bubbled with the humidified gas mixture (99.7% O_2, 0.3% CO_2), and a constant temperature of 23°C was maintained by a water bath. The incubation period was 2 h, after which the preparation was blotted and weighed exactly as before for gravimetric determination of the fluid transport rate. Final TEP was measured and then the contents of the sac were drained into a plastic micro-centrifuge tube for analysis of the final mucosal saline composition. The empty sac was then blotted dry and weighed. Subtraction of this weight from the initial and final filled weights yielded the initial (V_i) and final (V_f) mucosal saline volumes, respectively. Finally, the sac was cut open, and the gross area (A) of the exposed epithelial surface determined by tracing its outline onto graph paper (Grosell and Jensen, 1999). The mucosal saline was measured for changes in pH, P_{CO_2}, Cl^- and total CO_2, and total basic equivalent concentrations were calculated, as outlined below.

Bafilomycin experiments

In light of unexpected results with respect to freshwater *versus* seawater differences after feeding, *in vitro* gut sac experiments were carried out using the V-type H^+-ATPase inhibitor bafilomycin (LC Labs) to evaluate whether a proton pump was running in parallel to the HCO_3^- secretion mechanism. Experiments were performed at 1–3 h post-feeding on preparations from both freshwater and seawater animals (N=7–9 per treatment group) in the presence (1 μmol l^{-1} bafilomycin plus 0.1% DMSO, Sigma-Aldrich) or absence (0.1% DMSO only) of bafilomycin in the mucosal saline. Methods were identical to those in the preceding *in vitro* gut sac experiments, except that TEP was not measured.

Chyme sampling experiments

Killifish were held and maintained on a 1.5% daily ration as in the *in vitro* experiments. Feeding was suspended for 48 h, and then the fish were fed to satiation, followed by sacrifice for chyme sampling at 1–3 h post-feeding, 12–24 h post-feeding and after 7–14 days of fasting, as described above. The entire tract was quickly dissected out. At the 12–24 h and 1–2 weeks sampling times, the tract was essentially empty, and it was possible to obtain only a few microlitres of chyme at the entrance to the anterior intestine by gently massaging the tract in a posterior-to-anterior direction. At 1–3 h, the anterior and sometimes mid-intestine was generally full of food, and this was extruded through the anterior opening into a 0.5 ml centrifuge tube, again by gentle massage. The entire intestine was then cut open longitudinally, and the pH of the mucosa was measured by applying oesophageal micro-electrodes (see below) to the interior surfaces of the anterior, mid, posterior and rectal areas of the tract. At all sample times, chyme pH was also measured immediately after collection using the same micro-electrodes. At 12–24 h and 1–2 weeks, total CO_2 was measured directly on chyme samples, whereas, at 1–3 h, the food-rich chyme was centrifuged (2 min at 13,000 ***g***). The supernatant was measured immediately for pH and total CO_2, and then decanted for later analysis of Cl^-.

Analytical techniques and calculations

In the *in vivo* experiments, the initial and final water samples of each flux period were analyzed for titratable alkalinity and total ammonia concentrations, so as to calculate net acidic or basic equivalent flux, as outlined by McDonald and Wood (McDonald and Wood, 1981). Titratable alkalinity was determined by the original single end-point method of McDonald and Wood (McDonald and Wood, 1981), which was validated against the double end-point method (Taylor et al., 2007) in a recent study (Bucking and Wood, 2008). In brief, titratable alkalinity was determined by titration of 10 ml water samples to pH=4.0, using a Radiometer pHc 3005-8 combination electrode that was connected to a Radiometer pH meter PHM201. A Gilmont microburette was used to accurately deliver standardized acid (0.02 mol l^{-1} HCl) until the pH of the water sample fell below pH=5.0. The sample was then aerated for 15 min to remove CO_2, and the titration was then continued until a pH of 4.0 was reached. Water total ammonia concentrations were measured by the indophenol blue method (Ivancic and Degobbis, 1984). The net fluxes of ammonia (J_{Amm}; μmol kg^{-1} h^{-1}) and titratable alkalinity (J_{TAlk}; μmol kg^{-1} h^{-1}) were then calculated from the changes in concentration from initial to final samples, factored by volume, time and fish mass. The net acid–base flux ($J_{net H^+}$) was calculated as the difference between J_{TAlk} and J_{Amm}, signs considered (McDonald and Wood, 1981). A net acidic equivalent flux to the environment (i.e. a net flux of H^+) is shown by a positive value, whereas a net basic equivalent flux to the environment (i.e. a net flux of HCO_3^-) is shown by a negative value.

In the *in vitro* gut sac experiments, transepithelial potential (TEP, V) across the preparations at the start and end of the 2-h flux period was measured as described by Nadella and colleagues (Nadella et al., 2007). TEP was determined using agar/salt bridges (3 mol l^{-1} KCl in 4% agar) connected through Ag/AgCl electrodes (MicroElectrodes Inc.) to a Radiometer pHM 82 standard pH meter (Radiometer, Copenhagen, Denmark). All TEP values were recorded while the sacs were immersed in standard serosal saline, with mucosal reference at 0 mV. The mucosal side was accessed via the cannulation catheter and the serosal side via the outside bathing solution. Tip potential was routinely less than 2 mV, and the electrodes were checked for symmetry.

The initial and final samples of mucosal saline were analyzed for pH (Radiometer GK2401C glass combination electrode connected to a Radiometer PHM 71 blood-gas meter, calibrated with Radiometer precision buffers), Cl^- (chloridometer; CMT 10 chloride titrator, Radiometer) and total CO_2 (Corning 965 total CO_2 analyzer) concentrations. The P_{CO_2} and total basic equivalent concentrations ([HCO_3^-] + 2[CO_3^{2-}]) were calculated from the total CO_2 and pH measurements by means of the Henderson–Hasselbalch equation using the appropriate pK′ and pK″ values and CO_2 solubility coefficient at one-third strength seawater at 23°C. The pH dependence of pK′ was taken into account using the nomogram of Severinghaus (Severinghaus et al., 1956). Grosell and colleagues (Grosell et al., 1999) found that this method yielded results for total basic equivalent concentrations of intestinal fluid that were virtually identical to those determined using the double end-point titration method (Hills, 1973).

Fluid transport rates (μl cm^{-2} h^{-1}) were calculated from the difference between initial (V_i) and final (V_f) mucosal saline volumes, factored by the intestinal surface area (A, cm^2) and time (T, h). Absorption of Cl^- and secretion of basic equivalents (μmol cm^{-2} h^{-1}) were calculated as the difference between ($V_i \times X_i$) and ($V_f \times X_f$), factored by A and T, where X_i and X_f represent the initial and final

concentrations (nmol μl^{-1}), respectively, of the moiety in question (Grosell et al., 2005).

In the chyme sampling experiments, measurements of chyme pH, supernatant pH and mucosal surface pH were made using an oesophageal micro-electrode set (MicroElectrodes Inc.) connected to a Radiometer pHM 82 standard pH meter, again calibrated with Radiometer-precision buffers. There were never any significant differences between directly measured chyme pH and chyme supernatant pH, and so average values of the two measurements are reported. Analyses of total CO_2 and Cl^- concentrations, as well as calculations of P_{CO_2} and total basic equivalent concentrations in the chyme fluid, were performed as above.

Data have been reported as means ± s.e.m. (N=number of fish or gut sac preparations), unless otherwise stated. In a few cases, data were log-transformed so as to pass tests of normality (Shapiro–Wilk) and homogeneity (Bartlett's chi square) before further statistical analysis. In the *in vivo* study, temporal changes in J_{Amm}, J_{TAlk}, and $J_{net H^+}$ were examined with a repeated measures ANOVA, followed by Dunnett's test. A one-sample t-test was conducted to determine whether $J_{net H^+}$ values were significantly positive or negative in the *in vivo* studies, and a paired t-test was used to assess whether final acid–base conditions were significantly different from starting conditions in the mucosal saline in the *in vitro* incubations. For comparisons between treatments or sampling sites in the *in vitro* studies, one-way ANOVA was followed by a HSD post-hoc test (Tukey's honest significant difference) or a Bonferroni test, as appropriate. Differences between freshwater and seawater animals at specific times and sites were determined using an unpaired Student's t-test. Tests were two-tailed, and values were considered significantly different at P<0.05.

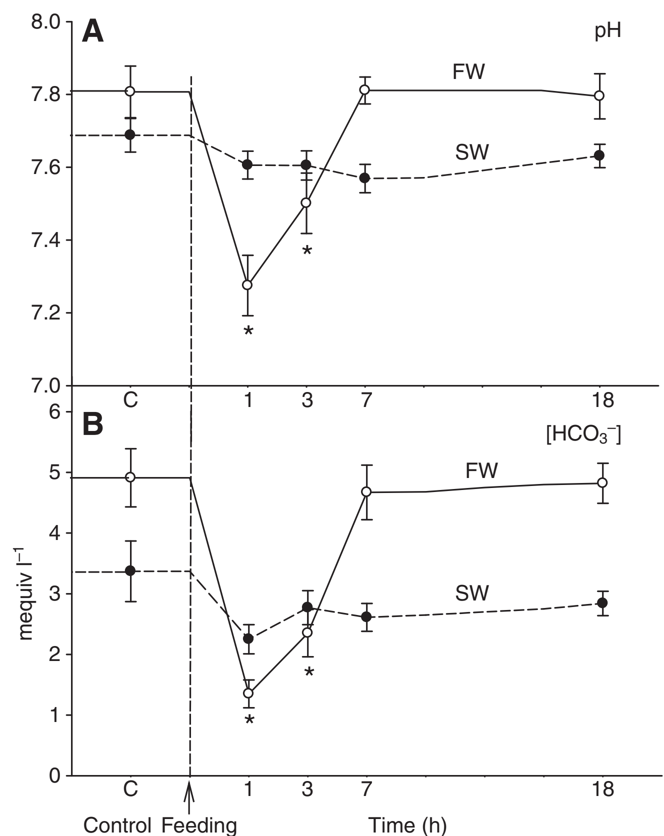

Fig. 2. Changes in blood 'metabolic' acid–base status following a satiation meal in *Fundulus heteroclitus* acclimated to either seawater (solid circles) or freshwater (open circles). Means ± 1 s.e.m. (N=7–9 at each point). See text for methodological details. Asterisks indicate means significantly different (P<0.05) from the corresponding control group at a particular time.

RESULTS

In vivo experiments

Before feeding, flux rates of ammonia (J_{Amm}; Fig. 1A) and titratable alkalinity (J_{TAlk}; Fig. 1A) were similar to one another in both freshwater and seawater killifish such that the animals were in approximate acid–base equilibrium with the environment: in both groups, net acid–base flux ($J_{net H^+}$) was not significantly different from zero (Fig. 1B). There was no difference between freshwater and seawater killifish in any of three flux rates at this time.

In seawater animals, J_{Amm} significantly increased by 2.6 fold immediately following feeding (i.e. at 0–4 h), but thereafter declined to levels that were not significantly elevated relative to the pre-feeding control period (Fig. 1A). At the same time, J_{TAlk} became more negative by 2.5 fold, but thereafter this response attenuated to levels that were not significantly different than those during the control period (Fig. 1A). These changes therefore cancelled each other out, such that there were no significant changes in $J_{net H^+}$ at any time after feeding in seawater killifish and overall acid–base flux remained not significantly different from zero (Fig. 1B).

In freshwater killifish, neither J_{Amm} (Fig. 1A) nor J_{TAlk} (Fig. 1A) changed significantly in the 24 h after feeding; therefore $J_{net H^+}$ remained close to zero (Fig. 1B). J_{TAlk} was significantly more negative in seawater fish than in freshwater fish at 0–4 h after feeding (Fig. 1A); there were no other significant differences between the two treatment groups at any time.

Blood sampling experiments

Under control conditions (i.e. after 48 h of fast), blood pH (when equilibrated to a fixed P_{CO_2}=2.25 Torr, Fig. 2A) was approximately 7.80 Torr in freshwater killifish, and 7.67 in seawater killifish, and the plasma HCO_3^- concentration (Fig. 2B) was correspondingly lower in the latter (4.9 versus 3.4 mequiv. l^{-1}). Only the difference in plasma HCO_3^- was significant, but, when the control and 18-h data were combined for each group, both pH and HCO_3^- were significantly different between the two salinities.

In seawater animals, feeding had no significant effect on the 'metabolic' acid–base status of the blood at any time, but, in freshwater animals, there was a marked metabolic acidosis at 1 h after the meal, with pH falling to 7.28 and HCO_3^- to 1.4 mequiv. l^{-1}. The acidosis was partially corrected at 3 h, and fully corrected by 7 h post-feeding (Fig. 2A,B).

In vitro experiments

In preparations from seawater killifish, rates of fluid absorption across the intestinal tract were highest at 1–3 h after feeding and had fallen to 29% by 12–24 h after the meal, thereafter rising to 55% after 1–2 weeks of fasting (Fig. 3A). These closely paralleled changes in net Cl^- absorption rates, which declined to 27% at 12–24 h after feeding, then increased to 56% after 1–2 weeks of fasting (Fig. 3B). For both variables, values at all three times were significantly different from one another.

Preparations from freshwater animals exhibited a generally similar pattern, but rates of both fluid and Cl^- absorption were notably higher than in seawater gut sacs at 1–3 h and 12–24 h after the meal (Fig. 3A,B). These differences were 1.50- and 1.63-fold at 0–3 h, and 2.08- and 2.59-fold at 12–24 h for fluid and Cl^- fluxes,

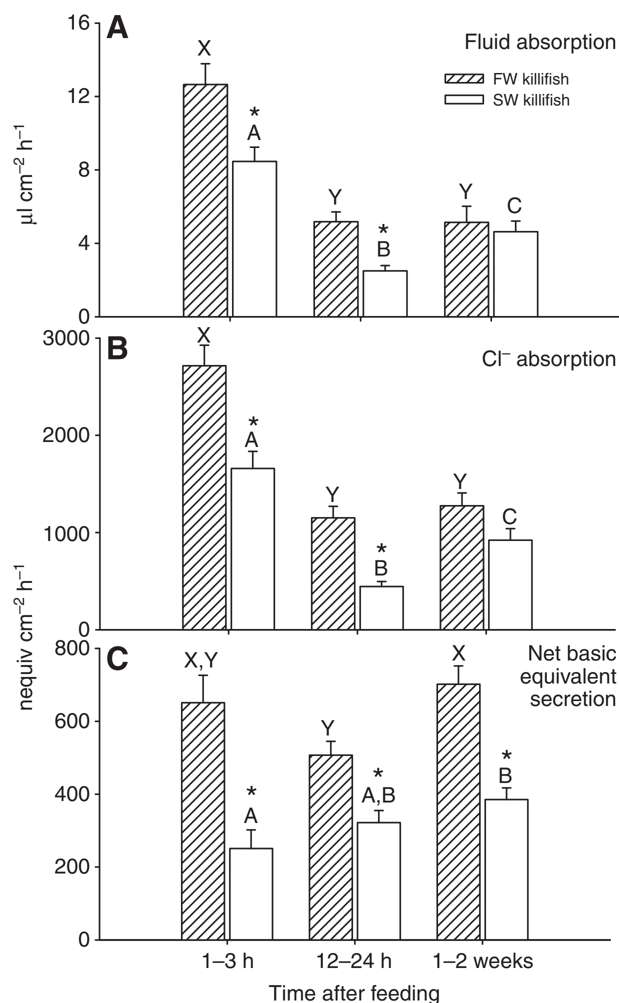

Fig. 3. Net rates of (A) fluid absorption, (B) Cl$^-$ absorption and (C) net basic equivalent secretion measured *in vitro* in gut sac preparations taken from *Fundulus heteroclitus* acclimated to either freshwater (cross-hatched bars) or seawater (open bars) at 1–3 h, 12–24 h (short-term fasting) and 1–2 weeks (long-term fasting) after a satiation meal. Means + 1 s.e.m. (*N*=19–27 for each of the six treatments). Within a salinity, means sharing the same letter are not significantly different (*P*>0.05). Asterisks indicate significant differences (*P*<0.05) between freshwater and seawater at the same time post-feeding.

Table 1. Concentration of Cl$^-$ (mmol l^{-1}) in the absorbate, as estimated from measured fluid and Cl$^-$ absorption rates of gut sac preparations taken from *Fundulus heteroclitus* acclimated to either freshwater or seawater, at various times after a satiation meal

	Freshwater	Seawater
1–3 h Post-feeding	225.5±9.6^{x} (25)	201.3±6.8^{a} (20)
12–24 h Post-feeding	242.6±18.5^{x,y} (21)	187.8±14.3^{a,*} (23)
1–2 weeks Post-feeding	286.2±24.4^{y} (19)	195.9±9.5^{a,*} (19)

Within a salinity, means sharing the same letter are not significantly different (*P*>0.05).
Asterisks indicate significant differences (*P*<0.05) between freshwater and seawater values at the same time post-feeding.
Means ± 1 s.e.m. (*N*).

respectively. After 1–2 weeks of fasting, there were no significant differences. In freshwater preparations, rates of fluid absorption fell to 41%, and Cl$^-$ absorption to 42%, at 12–24 h relative to the 1–3 h post-feeding values; however rates did not change significantly between 12–24 h and 1–2 weeks (Fig. 3A,B).

The Cl$^-$ concentration in the absorbate, calculated by dividing the Cl$^-$ absorption rate by the fluid absorption rate, was consistently higher in preparations from freshwater animals (significant at 12–24 h and 1–2 weeks), and increased with long-term fasting (Table 1). This did not occur in gut sacs from seawater killifish. Absorbate Cl$^-$ concentrations were in all cases higher than in luminal or serosal salines.

At all time-points, rates of net basic equivalent secretion were significantly higher in freshwater gut sacs than in seawater preparations (Fig. 3C). These differences amounted to 2.74 fold at 1–3 h, 1.66 fold at 12–24 h, and 1.89 fold at 1–2 weeks. Note that absolute rates of base secretion (Fig. 3C) were always much lower than those of Cl$^-$ uptake (Fig. 3B) and exhibited rather different temporal patterns. Therefore, the apparent net Cl$^-$:net base stoichiometry varied greatly with acclimation condition and time. In seawater gut sacs, base secretion rates increased progressively with time post-feeding, the difference becoming significant by 1–2 weeks, whereas, in freshwater preparations, only the moderate increase between 12–24 h and 1–2 weeks was significant (Fig. 3C).

All preparations started with the same pH (8.12±0.01), P_{CO_2} (2.51±0.02 Torr) and basic equivalent concentration (12.05±0.08 mequiv. l^{-1}; *N*=141) in the saline on both serosal and mucosal surfaces, and the serosal acid–base conditions did not change appreciably over the flux period, owing to the large external volume and constant gassing with the 0.3% CO_2/99.7% O_2 mixture. However, final acid–base conditions in the mucosal saline at the end of the 2-h incubation period varied greatly depending on the acclimation condition and time after feeding. In all cases, final pH (Fig. 4A), P_{CO_2} (Fig. 4B) and basic equivalent concentration (Fig. 4C) were significantly different from starting values (and serosal values), except for the 12–24 h post-feeding pH in the seawater killifish. Overall, these data illustrate that changes in mucosal pH were not solely attributable to differences in basic equivalent fluxes but also reflected contributions from differences in P_{CO_2}.

In preparations from seawater animals, final mucosal pH was depressed at 1–3 h post-feeding relative to starting values, unchanged at 12–24 h and elevated at 1–2 weeks (Fig. 4A). These changes correlated with the highest value of P_{CO_2} (almost 12 Torr) at 1–3 h, an intermediate value at 12–24 h and the lowest value at 1–2 weeks, although even the latter (approximately 4 Torr) was significantly elevated relative to starting values (Fig. 4B). Both final pH and final P_{CO_2} values in the mucosal saline differed significantly among the three sample times. Final basic equivalent concentrations did not differ significantly among the three times (Fig. 4C).

In preparations from freshwater animals, final mucosal pH was substantially higher than in seawater gut sacs at both 1–3 h and 12–24 h post-feeding, and at all times was significantly elevated relative to starting values (Fig. 4A). P_{CO_2} values in these freshwater gut sacs were again significantly elevated relative to starting values, but not to the same degree (e.g. approximately 6 Torr at 1–3 h) as in seawater preparations (Fig. 4B). Therefore freshwater P_{CO_2} values were significantly lower than the corresponding seawater values at 1–3 h and 12–24 h, but actually slightly higher at 1–2 weeks. Final basic equivalent concentrations were substantially higher than in

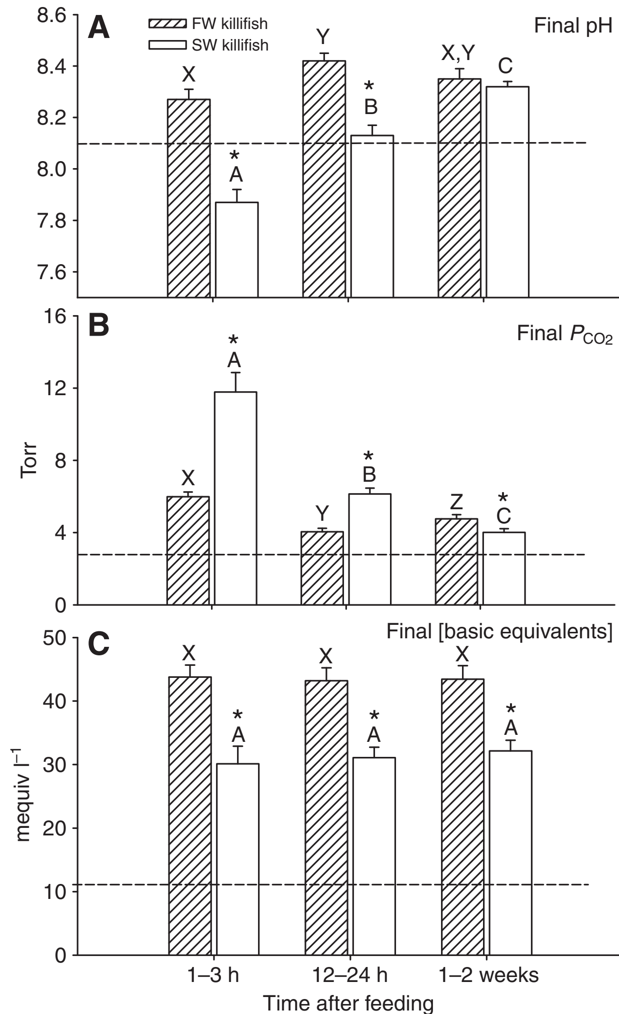

Fig. 4. Acid–base conditions in the mucosal saline measured *in vitro* at the end of 2 h of incubation in gut sac preparations taken from *Fundulus heteroclitus* acclimated to either freshwater (cross-hatched bars) or seawater (open bars), at 1–3 h, 12–24 h (short-term fasting) and 1–2 weeks (long-term fasting) after a satiation meal. (A) Final pH, (B) final P_{CO_2} and (C) final concentration of basic equivalents. Means + 1 s.e.m. (N=19–27 for each of the six treatments). Dotted lines indicate the starting conditions in the mucosal saline. Within a salinity, means sharing the same letter are not significantly different (P>0.05). Asterisks indicate significant differences (P<0.05) between freshwater and seawater preparations at the same time post-feeding.

seawater preparations, by 11–13 mequiv. l^{-1}, but did not differ significantly among the three times (Fig. 4C).

TEP was recorded relative to a mucosal reference of 0 mV, under conditions that were initially symmetrical but changed after 2 h owing to transport and metabolic processes of the preparations. Under all treatments, mean TEP values were generally low but positive (+0.8 to +4.8 mV; Fig. 5). At 1–3 h post-feeding, the initial values were significantly higher in preparations from seawater fish than in freshwater gut sacs, but this pattern reversed at 12–24 h. By 1–2 weeks, the values of both had fallen, and there were no significant differences. TEP values fell significantly from initial to final measurements in seawater preparations at 1–3 h and 12–24 h post-feeding but did not change significantly from initial to final values under the other treatment conditions.

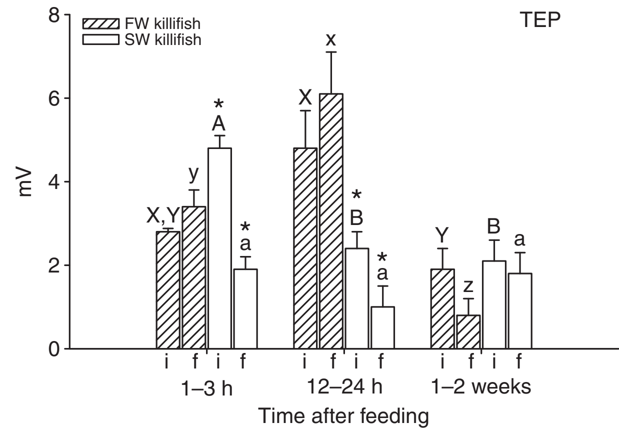

Fig. 5. Transepithelial potential (TEP) measured relative to a mucosal reference of 0 mV across *in vitro* gut sac preparations taken from *Fundulus heteroclitus* acclimated to either freshwater (cross-hatched bars) or seawater (open bars) at 1–3 h, 12–24 h (short-term fasting) and 1–2 weeks (long-term fasting) after a satiation meal. Values are shown separately for measurements taken at the start (i=initial) and end (f=final) of the 2-h incubation period Means + 1 s.e.m. (N=19–27 for each of the 6 treatments). Within a salinity, means sharing the same letter are not significantly different (P>0.05), with upper case letters used for initial values (i) and lower case letters used for final values (f). Asterisks indicate significant differences (P<0.05) between freshwater and seawater preparations at the same time post-feeding.

Bafilomycin experiments

The lower rates of net basic equivalent secretion in seawater preparations relative to freshwater preparations, especially at 1–3 h post-feeding (Fig. 3C), were unexpected but did agree with the fact that a metabolic acidosis was seen only in the blood of freshwater animals at this time (Fig. 2). Bafilomycin (1 μmol l^{-1}) was employed to evaluate whether the explanation was a proton pump running in parallel to the HCO_3^- secretion mechanism at different rates in the two salinities. Relative to DMSO-only controls, bafilomycin had no effect on rates of fluid absorption (Fig. 6A), Cl^- absorption (Fig. 6B), net basic equivalent secretion (Fig. 6C) or final mucosal P_{CO_2} (Fig. 6D) in freshwater gut sac preparations at 1–3 h post-feeding. However, in seawater preparations at this same time, bafilomycin treatment caused a significant 1.8-fold elevation of net basic equivalent secretion rate, raising it to a level not significantly different from that of freshwater preparations (Fig. 6C). Bafilomycin also attenuated the rise in mucosal P_{CO_2}, such that final P_{CO_2} became the same as in freshwater preparations (Fig. 6D). Notably, bafilomycin also increased the rates of both fluid (Fig. 6A) and Cl^- absorption (Fig. 6B) by 1.7–1.9 fold to levels much higher than those in freshwater preparations.

Chyme sampling experiments

Chyme samples were taken from intact fish at times matching those used in the gut sac experiments. At 1–3 h post-feeding, food was present in the anterior intestine, and sometimes also in the mid-intestine, but not in lower parts of the tract. At this time, there were substantial differences between seawater and freshwater animals (Fig. 7, Table 2), in agreement with the *in vitro* evidence for greater post-prandial uptake of Cl^- (cf. Fig. 3B) and secretion of basic equivalents (cf. Fig. 3C) in the freshwater fish. In seawater killifish, mean chyme Cl^- was more than twice as high as in freshwater fish (86.5±9.5 *versus* 40.7±3.6 mmol l^{-1}, N=7–8), a highly significant difference, whereas pH was approximately 1.0 unit lower (5.66

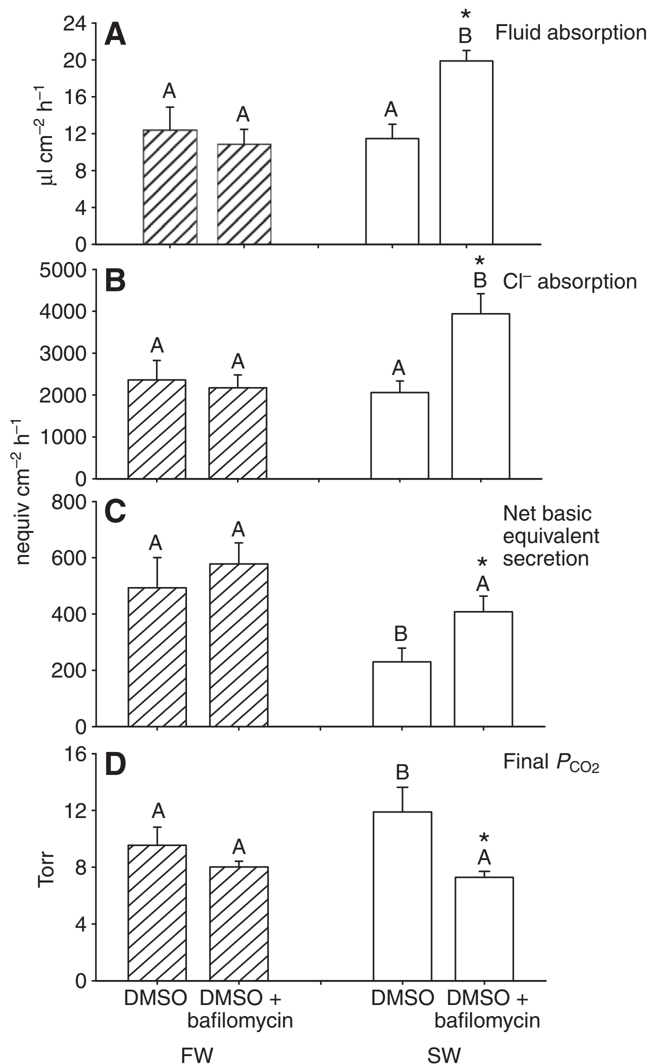

Fig. 6. The influence of the V-type ATPase inhibitor bafilomycin (1 μmol l^{-1} bafilomycin plus 0.1% DMSO) on net rates of (A) fluid absorption, (B) Cl$^-$ absorption, (C) net basic equivalent secretion and (D) final P_{CO_2} measured *in vitro* in gut sac preparations taken from *Fundulus heteroclitus* acclimated to either freshwater (cross-hatched bars) or seawater (open bars) at 1–3 h after a satiation meal. Means + 1 s.e.m. (N=7–9 for each of the four treatments). Means sharing the same letter are not significantly different (P>0.05). Asterisks indicate significant differences (P<0.05) between bafilomycin-treated preparations and those treated with the vehicle alone (0.1% DMSO).

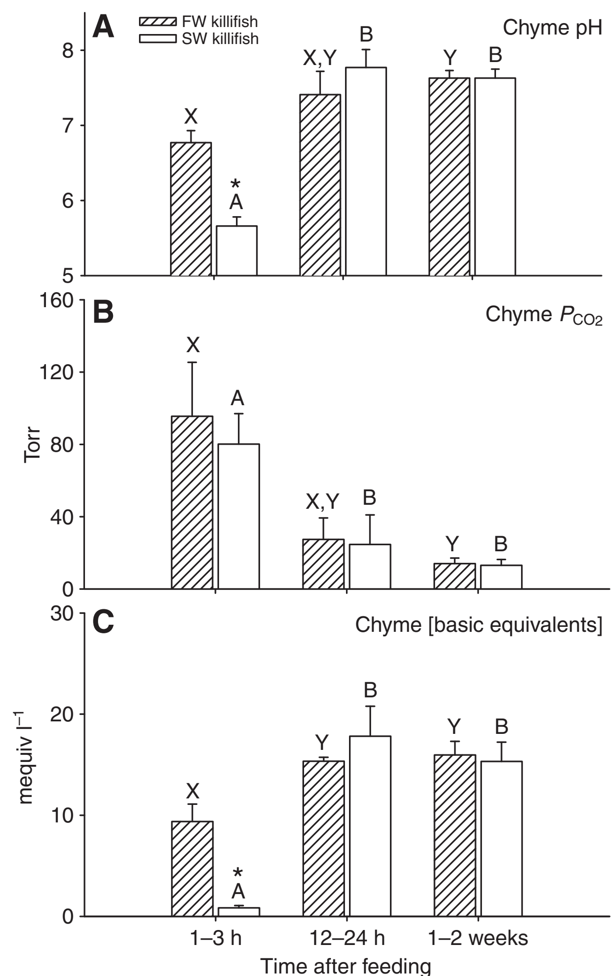

Fig. 7. Acid–base conditions in the chyme or intestinal fluid sampled from intact *Fundulus heteroclitus* acclimated to either freshwater (cross-hatched bars) or seawater (open bars), at 1–3 h, 12–24 h (short-term fasting) and 1–2 weeks (long-term fasting) after a satiation meal. (A) Chyme pH, (B) chyme P_{CO_2} and (C) concentration of basic equivalents in the chyme fluid. Means + 1 s.e.m. (N=6–8 for each of the six treatments). Within a salinity, means sharing the same letter are not significantly different (P>0.05). Asterisks indicate significant differences (P<0.05) between freshwater and seawater values at the same time post-feeding.

versus 6.77; Fig. 7A), and mean basic equivalent concentration (0.8 mequiv. l^{-1}) was less than 10% of the value (9.4 mequiv. l^{-1}) seen in freshwater fish (Fig. 7C). Remarkably, mean P_{CO_2} values in the chyme of seawater and freshwater fish, respectively, were 80 and 95 Torr (not significantly different; Fig. 7B). The total CO_2 and pH data from which these values are calculated are summarized in Table 2. Comparison with Fig. 7C reveals that only 20% (in seawater fish) and 65% (in freshwater fish) of total CO_2 was present as basic equivalents (i.e. HCO_3^-) at these pH values; the remainder existed as dissolved CO_2 in light of the very high P_{CO_2} values present in the chyme.

By 12–24 h post-feeding, the digestive tract had been cleared of food (except for occasional faecal pellets close to the rectum), and, in the small amount of intestinal fluid still present, acid–base parameters were no longer significantly different between freshwater and seawater killifish. In both groups, basic equivalent concentrations had risen substantially to approximately 17 mequiv. l^{-1}. Mean chyme pH values also increased to approximately 7.5, and P_{CO_2} values fell to approximately 25 Torr. After 1–2 weeks of fasting, there was no further significant change in the concentrations of basic equivalents, but intestinal fluid P_{CO_2} had fallen to approximately 13 Torr. More than 95% of total CO_2 was now present as basic equivalents (i.e. HCO_3^- and CO_3^{2-}).

When food pellets were incubated with two parts distilled water, so as to duplicate the consistency of 1–3 h chyme, pH was 6.03±0.01 (N=4), and total CO_2 was undetectable, and so the concentration of basic equivalents leached from the food and the P_{CO_2} were both essentially zero.

Measurements of mucosal surface pH revealed a clear anterior-to-posterior gradient, with pH increasing by approximately 0.5 units from

Table 2. Chyme fluid total CO_2 and pH, and intestinal surface pH values at various sites, from intact *Fundulus heteroclitus*, acclimated to either freshwater (FW) or seawater (SW), at various times after a satiation meal

		Chyme		Intestinal surface			
		Total CO_2 (mmol l^{-1})	pH	Anterior pH	Mid pH	Posterior pH	Rectum pH
1–3 h post-feeding	FW	14.53 ± 2.91	$6.77^{a,b}\pm0.16$	$6.61^{a}\pm0.15$	$6.78^{a,b}\pm0.14$	$7.03^{a,b}\pm0.10$	$7.12^{b}\pm0.10$
	SW	$4.37^{*}\pm0.67$	$5.66^{*,a}\pm0.12$	$6.05^{*,a}\pm0.17$	$6.29^{*,a}\pm0.16$	$6.33^{*,a}\pm0.16$	$6.88^{b}\pm0.12$
12–24 h post-feeding	FW	18.82 ± 1.85	$7.41^{a}\pm0.31$	$6.79^{b}\pm0.22$	$7.05^{a,b}\pm0.24$	$7.31^{a,b}\pm0.10$	$7.29^{a,b}\pm0.11$
	SW	19.43 ± 2.66	$7.77^{a}\pm0.24$	$7.06^{b}\pm0.15$	$7.30^{a,b}\pm0.16$	$7.51^{a}\pm0.12$	$7.59^{a}\pm0.16$
1–2 weeks post-feeding	FW	16.58 ± 1.35	$7.63^{a,b}\pm0.10$	$7.46^{a}\pm0.12$	$7.70^{a,b}\pm0.13$	$7.87^{a,b}\pm0.11$	$8.09^{b}\pm0.13$
	SW	15.91 ± 1.81	$7.63^{a,b}\pm0.12$	$7.47^{a}\pm0.16$	$7.65^{a,b}\pm0.16$	$7.88^{b}\pm0.14$	$7.97^{b}\pm0.19$

Within a salinity at one sample time, the means of pH values sharing the same letters are not significantly different ($P>0.05$).
Asterisks indicate significant differences ($P<0.05$) between freshwater (FW) and seawater (SW) values at the same time post-feeding.
Means ± 1 s.e.m. (N=6–8).

the anterior intestine to the posterior intestine and rectum (Table 2). In general, surface pH values followed the trends seen in chyme pH. Thus, in all sections except the rectum, they were significantly lower in seawater animals than in freshwater animals at 1–3 h post-feeding, whereas, by 12–24 h, absolute values were higher, and no longer significantly different between salinities. After 1–2 weeks of fasting, surface pH values in all sections had further increased, but again there were no significant differences between freshwater and seawater animals (Table 2). Notably, carbonate pellets, which are easily visible in other species, were not seen at any sample time in the intestinal tracts of either freshwater or seawater killifish.

DISCUSSION

Overview

Our results support two of our initial hypotheses, while disproving a third (see Introduction). With respect to our fourth hypothesis, differences seen *in vitro* were reflected qualitatively in measurements of chyme composition from intact animals at 1–3 h after feeding. First, in accord with our prediction, neither freshwater- nor seawater-acclimated killifish exhibited an elevated post-prandial base flux to the external water (Fig. 1), and neither exhibited a metabolic alkalosis in the blood (Fig. 2), reflecting the absence of digestive HCl secretion in this stomach-less species. However, the metabolic acidosis, after feeding, in the blood of freshwater killifish [and not in seawater killifish (Fig. 2)] is the first report, to our knowledge, of a post-prandial 'acidic tide'. Second, in isolated gut sacs, net Cl^- uptake was greater in freshwater-acclimated killifish than in seawater animals, particularly at 1–3 h and 12–24 h after feeding (Fig. 3B). However, contrary to prediction, net base secretion was lower in intestinal preparations from seawater-acclimated killifish at all times relative to freshwater gut sacs and was apparently not upregulated after feeding (Fig. 3C). Indeed the opposite trend was observed. Measurements of responses to bafilomycin explain this unexpected result, at least in part. Finally, our results indicate that the original observation of Babkin and Bowie (Babkin and Bowie, 1928) that *Fundulus heteroclitus* has an entirely alkaline digestive tract must be qualified (Table 2). Acid–base conditions in the chyme are remarkable (Fig. 7) relative to those normally thought to be present in other body fluids of fish.

The absence of a change in net acid–base fluxes after feeding

The finding that that $J_{\mathrm{net\,H^+}}$ remained unchanged after feeding in both freshwater and seawater killifish (Fig. 1) was in accord with the prediction, based on the absence of an HCl-secreting stomach (Babkin and Bowie, 1928) and therefore the absence of a systemic alkaline tide, as confirmed by measurements of blood 'metabolic' acid–base status (Fig. 2). Nevertheless, had net HCO_3^- secretion into the intestinal tract increased after feeding, a more positive $J_{\mathrm{net\,H^+}}$ (rather than the more negative $J_{\mathrm{net\,H^+}}$ recorded in marine sharks and freshwater trout – see Introduction) might have been expected. This did not occur. For seawater killifish, this result concurs with the observation that blood acid–base status was unaltered after feeding (Fig. 2), and the results of *in vitro* experiments showing that intestinal base secretion was actually depressed on a net basis at this time (Fig. 3C), a point that will be considered below. In freshwater killifish, the 'acidic tide' (Fig. 2) and higher net intestinal base secretion (Fig. 3C) suggest that a more positive $J_{\mathrm{net\,H^+}}$ flux into the water should have been seen after feeding. However, the apparent lack of direct Na^+ influx:acid efflux and Cl^- influx:base efflux linkage mechanisms at the gills when this species is in freshwater (Patrick et al., 1997; Patrick and Wood, 1999) might have limited or prevented a branchial response, thereby accentuating the blood acidosis.

The killifish is not alone in this pattern of unchanged acid–base flux after feeding, which has been seen in several other teleosts, but the explanation might be very different. The marine gulf toadfish (*Opsanus beta*) (Taylor and Grosell, 2006) and the euryhaline European flounder (*Platichthys flesus*) (Taylor et al., 2007) both exhibited a marked gastric acidification after feeding but showed no evidence of a systemic alkaline tide. In the latter study, this was true of both marine and freshwater specimens; acid–base flux to the water was also measured and, similarly, did not change. In one study on the freshwater rainbow trout (*Oncorhynchus mykiss*) (Cooper and Wilson, 2008), net acid–base flux to the water did not change after feeding, even though there was a marked systemic alkaline tide. In all of these cases, the general interpretation offered has been that enhanced secretion of basic equivalents by the intestine after feeding was sufficient to negate the alkaline tide and/or obviate the need for excretion of base to the external water. Bucking and colleagues (Bucking et al., 2009) evaluated this idea using an *in vitro* gut sac technique and showed that net intestinal base secretion rates were greatly elevated after feeding in seawater-acclimated trout, as postulated. Taylor and Grosell (Taylor and Grosell, 2009) provided similar evidence in the marine toadfish using an Ussing chamber approach. However, intestinal base secretion remained low and unchanged after feeding in freshwater-acclimated trout (Bucking et al., 2009), and so the explanation for freshwater fish with acid-secreting stomachs is uncertain.

Blood acid–base status in relation to salinity and feeding

In freshwater killifish, the present measurements of blood pH (~7.80) and plasma HCO_3^- concentration (4.9 mequiv. l^{-1}) are essentially identical to those reported by Patrick and colleagues (Patrick et al., 1997) for freshwater killifish using very similar techniques (they equilibrated the blood to a P_{CO_2} of 2.43 *versus* 2.25 Torr in the present study). The lower pH and plasma HCO_3^- levels under control conditions in seawater killifish (Fig. 2) are consistent with the moderate metabolic acidosis reported at higher salinities in numerous species, both euryhaline (e.g. Maxime et al., 1990; Wilson and Taylor, 1992) and stenohaline (e.g. Walker et al., 1989), and usually attributed to a modest reduction in the 'strong ion difference' as plasma Cl^- rises slightly more than plasma Na^+ (Stewart, 1981).

As noted above, the blood measurements confirm the lack of an alkaline tide after feeding in this agastric teleost, and the unchanged 'metabolic' acid–base status in seawater fish (Fig. 2) is consistent with the *in vitro* data (Fig. 3C). The post-prandial 'acidic tide' in the blood of freshwater killifish is unprecedented; it probably reflects a greatly elevated rate of intestinal base secretion at this time, combined with an inability to clear quickly the resulting acidic equivalent load at the gills (Patrick et al., 1997; Patrick and Wood, 1999). The *in vitro* measurements (Fig. 3C) did show a markedly higher net rate of basic equivalent secretion at this time relative to seawater fish, but it was not greatly elevated relative to other time-points in freshwater killifish. It is possible that, with the removal of neural and hormonal controls *in vitro*, the stimulation was attenuated.

Intestinal Cl^- uptake in relation to salinity and feeding

The *in vitro* gut sac experiments confirmed that Cl^- and fluid absorption increased after feeding (Fig. 3B). Marshall and colleagues (Marshall et al., 2002) also reported that intestinal Cl^- and fluid transport rates were greater after feeding, although their study was exclusively on gut sac preparations from seawater *Fundulus heteroclitus*. In the present study, this effect was more marked in freshwater fish and was corroborated by the lower Cl^- concentrations measured in the chyme of freshwater killifish at 1–3 h post-feeding. This finding fits with the gut sac results of Scott and colleagues (Scott et al., 2006) showing that Cl^- uptake by the intestinal tract of *Fundulus heteroclitus* is upregulated during acclimation to freshwater. The capacity for Cl^- uptake by the killifish gut is clearly very high. Assuming that a 5 g animal has an intestinal surface area of approximately 10 cm^2, then the peak Cl^- uptake rate measured after feeding (Fig. 3B) would amount to about 5600 nmol g^{-1} h^{-1}. This would be more than adequate to replace measured branchial Cl^- loss rates of 200–800 nmol g^{-1} h^{-1} in freshwater killifish, which lack an active Cl^- uptake mechanism at the gills (Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003). However, these *in vitro* rates probably overestimate *in vivo* rates, where Cl^- availability in the food might be limiting and feeding is periodic, in contrast to the almost limitless supply provided by the mucosal saline. In future experiments, it will be of interest to quantify how much Cl^- the freshwater killifish actually acquires from the diet.

These high rates of Cl^- absorption are likely accompanied by Na^+ absorption and HCO_3^- exchange (reviewed by Grosell, 2006; Grosell and Taylor, 2007). The calculated Cl^- concentration of the absorbate was higher in gut sacs from freshwater fish (Table 1), suggesting that intestinal permeability is modulated so as to favour ion absorption over water absorption in accord with the requirements of osmoregulation in freshwater. This effect became more pronounced during long-term fasting in freshwater as the need for Cl^- became more severe. To our knowledge, this is the first time that these adaptive shifts in Cl^- *versus* water transport with respect to osmoregulatory needs have been documented. In all treatments, absorbate Cl^- concentrations (188–286 mmol l^{-1}; Table 1) were higher than blood plasma Cl^- concentrations of *Fundulus heteroclitus* [approximately 135 mmol l^{-1} in both freshwater- and seawater-acclimated animals (Scott et al., 2006; Scott et al., 2008)] and higher than luminal Cl^- concentrations. This supports the view that the absorbate is hyperosmotic, and the values are in line with those reported for other species in the review of Grosell (Grosell, 2006). However, the data of Marshall and colleagues (Marshall et al., 2002) for seawater killifish indicated an isotonic absorbate Cl^- concentration of approximately 133 mmol l^{-1}; the reason for this difference is unknown.

In addition to Cl^-:HCO_3^- exchange, Na^+-coupled Cl^- uptake pathways might become more important after feeding, as well as Na^+-coupled nutrient absorption (glucose in the present experiments). Indeed, all three pathways might be involved in promoting fluid uptake at this time. Generally higher TEP values were seen after feeding (Fig. 5). Na^+–glucose co-transport is electrogenic (Umbach et al., 1990) and might explain this effect. The present TEP values are slightly more positive than reported for most other teleost intestinal preparations, where values are very close to zero under symmetrical conditions, although most previous data have been obtained from fasted animals [summarized by Loretz (Loretz, 1995)].

Intestinal base secretion in relation to salinity and feeding, and responses to bafilomycin

Contrary to expectations, the rate of secretion of basic equivalents *in vitro* was higher in gut sacs from freshwater killifish than from seawater specimens (Fig. 3C). This is not to say that rates in seawater preparations were low, as they were in the range reported for many other marine fish (Wilson et al., 2002; Wilson and Grosell, 2003; Grosell et al., 2005; Grosell et al., 2009a; Grosell, 2006; Grosell and Taylor, 2007; Bucking et al., 2009; Taylor and Grosell, 2009). Rather, freshwater rates were unexpectedly high. As there is no need to precipitate Ca^{2+} and Mg^{2+} from the intestinal fluids in freshwater fish, we suggest that these base secretion rates are high either to facilitate the uptake of Cl^- or to promote some aspect of the digestive process. However, the latter seems less likely as base secretion rates did not increase immediately after feeding in freshwater fish and actually declined after feeding in seawater (Fig. 3C). The *in vivo* measurements support this view (Fig. 3C). It could be argued that, as there is no elevation of HCl secretion after feeding owing to the absence of an acid-secreting stomach, there is no need for greater intestinal HCO_3^- secretion at this time, in contrast to *in vitro* measurements in seawater preparations (Bucking et al., 2009; Taylor and Grosell, 2009) and *in vivo* data from other teleosts with gastric HCl secretion (Taylor and Grosell, 2006; Taylor et al., 2007). But why should base secretion rates decrease after feeding? The answer might lie in the fact that it is possible to measure only the *net* secretion of basic equivalents.

In the seawater trout and toadfish, Grosell and colleagues (Grosell et al., 2007; Grosell et al., 2009a; Grosell et al., 2009b) have recently presented evidence that a V-type H^+-ATPase working in co-operation with membrane-associated carbonic anhydrase runs in parallel with Cl^-:HCO_3^- exchange at the apical surface of the enterocytes. The mechanism serves to maintain the gradient for HCO_3^- secretion, limit cellular acidification and prevent a rise in luminal HCO_3^- concentration (and therefore osmolality) above that needed to precipitate Ca^{2+} and Mg^{2+}. When this mechanism was blocked by bafilomycin in fasted trout and

toadfish, the net secretion rate of basic equivalents increased by 10–30% (Grosell et al., 2009a; Grosell et al., 2009b). The results of the present experiments indicate that H^+-ATPase plays an even larger role immediately after feeding in seawater killifish because the net secretion rate of basic equivalents *in vitro* was increased by 80% in response to bafilomycin, raising the value to a level similar to that seen in freshwater preparations (Fig. 6C). By contrast, bafilomycin had no effect in freshwater preparations; a simple interpretation is that the H^+-pump is silenced in freshwater animals, whereas in seawater animals it short-circuits part of the net base secretion. This explains the lower measured *net* base secretion rate, and the lower final pH and higher P_{CO_2} both in the mucosal saline *in vitro* (Fig. 4A,B) and in the chyme *in vivo* (Fig. 7A,B). Note that bafilomycin attenuated the rise in P_{CO_2} *in vitro* in seawater preparations, reducing it to the level seen in freshwater gut sacs (Fig. 6D).

The rate of net base secretion was always less than the rate of net Cl^- uptake, but the apparent Cl^-:base stoichiometry varied with treatment and time post-feeding (Fig. 3B,C). At 12–24 h and 1–2 weeks, the ratio was approximately 2 in both freshwater and seawater preparations. This is in accord with data summarized by Grosell (Grosell, 2006) from various marine teleosts, all of which were fasted, indicating that HCO_3^- exchange accounted for a significant fraction of Cl^- uptake. However, when Cl^- absorption rates were increased at 1–3 h after the meal in the present study, the Cl^-:base ratio increased to 4 in freshwater animals and 6 in seawater animals (Fig. 3B,C). While this might suggest that anion exchange becomes less important for overall Cl^- uptake post feeding, the results of the bafilomycin experiments demonstrate that this is an oversimplification. At least in seawater killifish, the observed increase in net Cl^-:net base ratio appears to reflect increased apical proton extrusion (Fig. 6C), which would reduce the overall *net* rate of base secretion.

Grosell and colleagues (Grosell et al., 2009a; Grosell et al., 2009b) argued that this mechanism would be beneficial in a seawater teleost because it would promote water absorption by a reduction in luminal osmotic pressure, although fluid fluxes were not measured in those studies. However, the current experiments indicate exactly the opposite: bafilomycin greatly increased fluid absorption (Fig. 6A), and this was probably linked to greatly increased Cl^- absorption (Fig. 6B). At present, the explanation for this phenomenon is not clear. Presumably, the resulting change in intracellular acid–base status and/or apical membrane potential in the enterocytes favors non-HCO_3^--linked transport processes for Na^+, Cl^- and water uptake.

Remarkable acid–base conditions in the chyme

Using only litmus paper, Babkin and Bowie (Babkin and Bowie, 1928) reported that the pH of the digestive tract of fasted seawater *Fundulus heteroclitus* was always alkaline (8.0–9.2); after ingestion of a variety of foods, chyme pH fell slightly but remained alkaline. Using modern technology, we found lower pH values that were generally closer to 7.0 in marine and freshwater specimens subjected to both short- and long-term fasting, as well as in freshwater animals at 1–3 h post-feeding (Table 2, Fig. 5A). Interestingly, carbonate pellets, which are easily visible in other species (e.g. Wilson et al., 1996; Wilson, 1999; Grosell, 2006), were not seen at any sample time in the intestinal tracts of either freshwater or seawater killifish. It is possible that some dispersed precipitates of calcium and magnesium carbonate salts might have been present, but the absence of distinct aggregates probably reflects the less alkaline conditions seen in the killifish tract. In some species, basic equivalent concentrations can reach 100 mequiv. l^{-1} and pH values close to 9.0, in contrast to the more moderate conditions in the killifish intestine (Fig. 7, Table 2).

In seawater killifish, the tract became decidedly acidic after feeding (5.66 in chyme, 6.05 at the surface of the anterior intestine), and P_{CO_2} became very high (80–95 Torr) at both salinities. Mucosal fluid P_{CO_2} was also elevated *in vitro* (and mucosal pH depressed), but to a lesser extent (6–12 Torr; Fig. 4A,B). As there was substantial secretion of basic equivalents *in vitro* at this time (Fig. 3C, Fig. 4C), but low concentrations *in vivo* (indeed almost none in seawater fish; Fig. 7C), it seems likely that a substantial fraction of the HCO_3^- secretion was titrated by simultaneously secreted H^+ ions (Grosell et al., 2009a; Grosell et al., 2009b), at least in seawater killifish (Fig. 6C), and converted into high levels of dissolved CO_2 (i.e. elevated P_{CO_2}; Fig. 4B, Fig. 6D) in the chyme fluid (Fig. 7B). Such high levels of P_{CO_2} are unprecedented in the body fluids of water-breathing fish, where values are usually in the 1–5 Torr range. Is this phenomenon peculiar to the killifish?

There are now a large number of papers reporting intestinal fluid total CO_2 and pH measurements from fasted marine fish (e.g. Wilson, 1999; Wilson et al., 1996; Wilson et al., 2002; Wilson and Grosell, 2003; Grosell et al., 1999; Grosell et al., 2005; Grosell et al., 2007; Grosell et al., 2009a; Grosell et al., 2009b; Grosell, 2006; Grosell and Taylor, 2007; Genz et al., 2008), and from these we calculate intestinal P_{CO_2} values uniformly in the 1–5 Torr range. To our knowledge, there are only three other studies reporting comparable measurements from recently fed fish, and from these we calculate intestinal fluid P_{CO_2} values as high as 13–25 Torr in the gulf toadfish (Taylor and Grosell, 2006), 6–8 Torr in the European flounder (Taylor et al., 2007) and approximately 20 Torr in the spiny dogfish, a marine elasmobranch (Wood et al., 2007b). Thus, high P_{CO_2} values might be characteristic of the digestive process in fish, but particularly marked in killifish because of the absence of a stomach. Possible advantages for digestive enzyme activities and other processes remain to be explored but could include diffusion of CO_2 into enterocytes, where hydration mediated by intracellular carbonic anhydrase would facilitate further anion exchange and thus Cl^- uptake, as suggested recently (Grosell et al., 2009a). In addition, one obvious benefit is that these high P_{CO_2} values should greatly increase the release of O_2 from blood passing through the intestinal mucosa, thereby fuelling the many transport processes that are active during digestion and absorption. In this regard, the blood of *Fundulus heteroclitus* exhibits a robust Bohr shift (Mied and Powers, 1978; DiMichele and Powers, 1982), which should facilitate this O_2 release.

ACKNOWLEDGEMENTS

Supported by an NSERC Discovery Grant to C.M.W. and NSF IOB 0743903 to M.G. C.M.W. is supported by the Canada Research Chair Program. We thank two anonymous reviewers and the Editor whose constructive comments led to additional informative experiments. The experiments were approved by institutional animal care committees at the University of Miami and McMaster University.

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