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Intestinal bicarbonate secretion in marine teleost fish—source of bicarbonate, pH sensitivity, and consequences for whole animal acid–base and calcium homeostasis

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Abstract

Whole animal studies using seawater European flounder (*Platichthys flesus*) revealed that increasing intestinal [Ca^{2+}] to 20 mM stimulated net HCO_3^- base secretion by 57%, but this was effectively balanced by an increase in net acid secretion, likely from the gills, to maintain whole animal acid–base status. Higher Ca^{2+} concentrations (40 and 70 mM) in ambient seawater resulted in reduced plasma total CO_2. This indicates (1) imperfect acid–base compensation, and (2) that endogenous metabolic CO_2 is insufficient to fuel intestinal HCO_3^- secretion, under hyper-stimulated conditions. Bicarbonate secretion plays an important role in preventing calcium absorption by precipitating a large fraction of the imbibed calcium as $CaCO_3$. Indeed, under high Ca^{2+} conditions (20 mM), up to 75% of the intestinal Ca^{2+} is precipitated as $CaCO_3$ and then excreted. This is undoubtedly important in protecting the marine teleost kidney from the need for excessive calcium excretion and risk of renal stone formation. Using an in vitro pH-stat technique with the isolated intestinal epithelium, the replacement of serosal CO_2 with a HEPES buffered saline had no effect on HCO_3^- secretion, indicating that the endogenous supply of HCO_3^- from CO_2 hydration within epithelial cells is adequate for driving baseline secretion rates. Further, in vitro data demonstrated a stimulatory effect of low pH on intestinal HCO_3^- secretion. Thus, both luminal Ca^{2+} and H^+ can regulate HCO_3^- secretion but the precise mechanisms and their potential interaction are currently unresolved.

Keywords: Calcium carbonate precipitation; Osmoregulation; Water absorption; Chloride–bicarbonate exchange; Calcium and magnesium homeostasis; Acid–base balance

1. Introduction

Teleost fish hypo-osmoregulate in the marine environment, so they are constantly faced with osmotic water loss and passive gain of many different ions. To avoid dehydration they drink the ambient seawater, absorb the majority of the imbibed volume within the intestine, and the remainder is excreted as rectal fluid [1–3]. Walsh et al. [4] were the first to report that the imbibed seawater becomes alkaline (pH 8.4–9.0) and rich in HCO_3^- ions (40–130 mM) as it transits along the intestine (see also Refs. [5–9]). The high luminal HCO_3^- concentration is the product of apical Cl^-/HCO_3^- exchange [5,7,10], which seems to contribute significantly to net Cl^- and thereby fluid absorption. An additional functional significance of such high concentrations of HCO_3^- appears to lie in the abundance of divalent cations in the seawater that marine teleosts drink. The combination causes Ca^{2+} and Mg^{2+} to precipitate as insoluble carbonates which are then excreted. The precipitation appears to facilitate water absorption by effectively removing osmolytes (primarily calcium) from the intestinal lumen that would otherwise accumulate and retard osmotic water absorption into the blood. The HCO_3^- secretion therefore plays a rather novel role in osmoregulation [9]. Furthermore, an important stimulant of intestinal HCO_3^- secretion, at least in the flounder, appears to be specifically Ca^{2+} (rather than Mg^{2+}) entering the intestine both in vivo and in vitro [9].

Another potential function of the precipitation process could be to limit the intestinal absorption of calcium. Marine

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teleosts live in a hyper-calcaemic environment and hence face a continuous tendency towards calcium uptake. The normal route for excretion of excess divalent ions in marine teleosts is the kidney [3,11–13], but urine flow rates are notoriously low [14,15] and urine calcium cannot become too concentrated if solubility limits and kidney stones are to be avoided. Thus, it makes physiological sense for marine teleosts to limit the initial uptake of calcium from seawater as far as possible, to minimise the need for excess calcium excretion via the susceptible renal system.

The precipitation of $CaCO_3$ is facilitated by the strongly alkaline luminal environment within the intestine (pH as high as 9.0; [7,8]). It would thus appear that any retardation of this alkalinisation would result in a reduction of precipitate formation as was recently observed in European flounder [9]. In the same study a tendency towards increased intestinal HCO_3^- secretion was observed when intestinal fluids were buffered to maintain lower luminal pH, suggesting that this secretion process may be stimulated by reduced pH as well as calcium. However, the pH sensitivity of intestinal bicarbonate secretion has not yet been fully characterised.

The present study investigated the potential role of calcium carbonate precipitation within the intestine on whole animal Ca^{2+} handling by manipulating either the availability of intestinal Ca^{2+} or limiting the alkalinisation potential using HEPES-buffered intestinal salines (pH 7.5). Further, the impact of stimulating intestinal HCO_3^- (base) secretion upon whole animal acid–base balance was investigated. Very high ambient Ca^{2+} concentrations in vivo resulted in reduced plasma total CO_2 indicating less than perfect compensation for elevated intestinal base secretion. This raised the question of whether endogenous metabolic intestinal CO_2 was sufficient to fuel the intestinal HCO_3^- secretion or whether exogenous (plasma) CO_2 is required to fuel net HCO_3^- base secretion. A pH-stat in vitro approach using isolated intestinal epithelia was therefore employed to address the questions of sources of secreted HCO_3^- and the potential pH-sensitivity of this process.

2. Materials and methods

2.1. Animal source and holding

European flounder (*Platichthys flesus*; 150–1500 g) for the intestinal perfusion and pH-stat experiments were obtained from a commercial fishing boat fishing in the northern part of the Sound of Copenhagen Denmark and held in a recirculating artificial seawater system at 30 ppt salinity and 13 ± 1 °C at the August Krogh Institute, Copenhagen, as described previously [9]. Fish were not fed after capture to eliminate the contamination of intestinal fluids with faecal matter and to allow stabilisation of standard metabolic rate. European flounder (150–450 g) for a high calcium-seawater experiment were obtained from a commercial supplier (Aquarium Technology, Dorset, UK), and held in a recirculating natural seawater system at 33 ppt salinity and 13 ± 1 °C at the Department of Biological Sciences, University of Exeter.

2.2. In vivo intestinal perfusion procedures

To prepare for surgery, flounder (mean mass 318 ± 15 g, $n=21$) were anaesthetised in a 100 mg l^{-1} solution of tricaine methanesulfonate (MS222; Sigma) in seawater and then maintained on a wet table with the gills constantly irrigated with an aerated solution of this anaesthetic throughout surgery. A blood catheter was implanted in the caudal artery or vein and filled with Cortland saline [16] containing 50 i.u. ml^{-1} sodium heparin (Sigma). Fish were then prepared for in situ perfusion of the intestine according to the method adopted for other flatfish [5,17,18] and described in detail in Ref. [9] with an infusion catheter inserted at the junction between the stomach and the anterior intestine, and a stomach drain catheter inserted to allow any imbibed seawater to flow directly from the stomach to the external medium. Each fish was then fitted with a rectal catheter/collection bag as described in Ref. [9]. All incisions were treated with antibiotic (oxytetracycline) prior to closure to prevent infection.

Following surgery, flounder were placed in individual well-aerated flux chambers (2 or 5 l depending on fish size) continuously supplied with sea water at ~ 500 ml min^{-1}. During the recovery of ventilatory activity, the intestinal catheter was connected to a peristaltic pump (Gilson, Minipuls 3) and perfused with one of three gut saline solutions (see below). A perfusion rate of 3.80 ± 0.16 ml kg^{-1} h^{-1} ($n=21$) was used (in excess of the normal drinking rate of 2.02 ± 0.36 (6) ml kg^{-1} h^{-1} in flounder from a previous study acclimated to 30 ppt seawater), to ensure an adequate supply of fluid volume to the intestine for osmoregulation. Each flounder was then continuously perfused with the appropriate saline for 72 h, and blood samples were taken after 12 and 36 h for the first three fish in each group, and after 60 h for all the fish. During the 3-day experiments, the fluxes of acid–base equivalents between the fish and the seawater external medium were measured every day. For these fluxes the flow of seawater into each chamber was turned off for a 22-h period and 50-ml water samples taken at the start and end of each of these static periods. Between these 22-h flux periods the flow of seawater to each flux chamber was restored for 2 h to allow restoration of background seawater conditions (i.e. to flush out accumulated ammonia, etc.).

2.3. Perfusion treatments

Flounder were allocated to one of three experimental groups ($n=7$ per group) that were perfused with one of three different intestinal salines. These salines were designed to determine how much the presence of calcium in the intes-

tinal fluids influences (a) bicarbonate secretion (already reported elsewhere [9]) and ultimately the precipitation of insoluble calcium carbonate, (b) whole animal acid–base balance and (c) the net and unidirectional absorption of calcium into the blood. The control saline (see Table 1) contained 5 mM Ca^{2+} similar to the level found in vivo in rectal fluid. The second group was perfused with saline with an elevated calcium concentration (20 mM) to enhance the potential for calcium absorption or precipitation as carbonate. The additional 15 mM calcium was added as $CaCl_2$ and the $MgCl_2$ was reduced by the same amount to maintain a constant chloride concentration and osmolality in this saline (Table 1). The third saline contained the normal calcium concentration (5 mM) but had its buffer capacity enhanced by 100-fold by the addition of 5 mM instead of 0.05 mM HEPES (pH 7.4). This was designed to limit the ability of the intestine to alkalinise the luminal fluid and hence form CO_3^{2-} ions, which in turn were expected to minimise the precipitation of $CaCO_3$ caused by intestinal bicarbonate secretion. All salines were exactly matched with respect to osmolality by the addition of an appropriate amount of mannitol prior to use in perfusions. All salines were initially pH 7.4 and bicarbonate-free such that any alkalinisation and bicarbonate appearing in the rectal fluid must have been derived from intestinal secretion. All salines were additionally spiked with ^{45}Ca (Amersham) to achieve a specific activity of 3.3 μCi μmol^{-1} (giving approximately 45,000 and 180,000 cpm ml^{-1} for 5 and 20 mM calcium salines, respectively).

At the end of experiments, the perfusion catheter was carefully detached from the peristaltic pump and flounder were exanguinated via the blood catheter while still within the flux chambers. This blood was subsequently used for counting for ^{45}Ca activity. Fish were then removed from the flux chamber and rinsed in fresh water prior to severing the spinal chord and destruction of the brain. Once the integrity of the rectal catheter/bags was checked for leaks (any leaking bags were rejected from the experiment), their contents were transferred to 50-ml capped tubes and weighed to assess the volume of fluid retrieved. The body cavity was then dissected open and all urine present in the bladder was collected using 5-ml syringes and 23-G needles and transferred to vials. The intestine was then tied off at the anterior and posterior ends, dissected out, and the entire contents emptied into vials. The fluid and mucus within these intestinal samples were analysed separately as for the above rectal bag collections.

Table 1
Nominal composition of salines used for intestinal perfusion

	Control	High Ca^{2+}	HEPES buffered
Na^+	50	50	50
Mg^{2+}	115	100	115
Ca^{2+}	5	20	5
K^+	5	5	5
Cl^-	140	140	140
SO_4^{2-}	77.5	77.5	77.5
Mannitol	4.95	4.95	0
HEPES (pH 7.4)	0.05	0.05	5
Osmolality	308	308	308

Concentrations of inorganic ions, mannitol and HEPES are in mM; osmolality is in mosM kg^{-1}.

2.4. In vivo flounder exposure to elevated ambient calcium in seawater

An experiment was performed with non-cannulated flounder to examine whether additional calcium added to the ambient seawater would up-regulate intestinal bicarbonate excretion to the extent that it could alter blood total CO_2 content. In this experiment, 12 flounder were divided into three groups and transferred to one of three experimental treatments. The control group was transferred to a 50-l tank of natural seawater (33.9 ± 0.5 ppt, $N=4$) containing 10 mM calcium. The remaining fish were transferred to two 50-l tanks of natural seawater supplemented with $CaCl_2$ to give final calcium concentrations of either 40 or 70 mM. For these two tanks, deionised water was also added until the total salinity matched that of the control tank (40 mM $CaCl_2$ tank = 33.2 ± 0.5 ppt, $N=4$; 70 mM $CaCl_2$ tank = 33.7 ± 0.2 ppt, $N=4$). As a result, in the two experimental tanks the seawater had elevated calcium, almost identical chloride and total salinity, but proportionally lower concentrations of all other seawater ions.

Flounder were left for 72 h in these three media, and then individually anaesthetised in 0.5 g l^{-1} MS222 (adjusted to pH 8 with NaOH) before weighing and sampling blood (by caudal puncture) into heparinised syringes and intestinal fluid as described previously [7].

2.5. In vitro procedures—pH-stat experiments

The pH-stat experiments were carried out as previously described [9]. In brief, isolated segments from the anterior intestine were mounted in a custom made glass Ussing chamber exposing 2.72 cm^2 of intestine to a bath volume of 12 ml. The mucosal side of the chamber was equipped with a glass pH-electrode (pHG200, Radiometer, Copenhagen) and an Ag/AgCl-reference electrode with a K_2SO_4 outer liquid junction (REF251, Radiometer). This side was also fitted with a burette tip delivering titrant (20 mM HCl) via an autoburette (ABU 901, Radiometer) coupled to an automatic pH-stat controller (PHM290, Radiometer). To obtain stationary titration curves, the mucosal saline was unbuffered (see Table 2) and gassed with 100% O_2 to allow for the volatile CO_2 arising from HCO_3^- titration and epithelial respiration to escape the mucosal solution. In addition to mixing by gassing, both half-chambers were mixed by magnetic stirrers. The entire experimental setup was placed in an environmental chamber maintained at 13 °C to match the conditions under which the fish were held prior to experimentation. The intestinal epithelium was equilibrated under current clamp (0 μA) conditions while the transepithelial potential was

Table 2
Composition of salines used in pH-stat experiments

	Serosal (HCO_3^-/CO_2) (mM)	Serosal (HEPES) (mM)	Mucosal (mM)
Na^+	145.9	145.9	50
Mg^{2+}	1.5	1.5	100
Ca^{2+}	6.0	6.0	5
K^+	2.5	2.5	5
Cl^-	139.5	139.5	110
SO_4^{2-}	1.5	1.5	77.5
PO_4^{3-}	3.0	3.0	
HCO_3^-	11.9		
HEPES		11.9	
(mosM/kg)	283	283	281.5

recorded (Amp, Physiologic Instruments VCC600). The transepithelial potential (TEP) typically stabilized after 1–1.5 h, after which pH-stat titration was commenced. Prior to and after pH-stat titrations, pulses (50 μA) of 1-s duration every 60 s allowed for calculation of epithelial conductance and resistance according to Ohm's law from the resulting change in potential difference. Pulsing is not possible during pH-stat titrations as it interferes with the pH-measurements. Tissue preparations exhibited stable electrophysiological parameters for up to 10 h.

Initial experiments testing the effect of symmetrical (serosal saline on both sides of the epithelium) versus asymmetrical conditions on electrophysiological parameters revealed substantial differences in TEP. All subsequent experiments were conducted under asymmetrical conditions to mimic in vivo conditions best possible.

2.6. Source of HCO_3^-?

Because stimulated intestinal HCO_3^- secretion in vivo in response to elevated luminal Ca^{2+} resulted in reduced plasma total CO_2, experiments were performed to investigate the source of secreted HCO_3^-. In control experiments, the intestinal epithelium was exposed to a HCO_3^-/CO_2 buffered serosal saline gassed with 0.5% CO_2 in O_2 to mimic in vivo conditions (see Table 2). Experiments were conducted with HEPES buffered CO_2-free serosal solutions to test if transepithelial HCO_3^- transport occurred and if serosal CO_2 might act as substrate for epithelial carbonic anhydrase providing for intestinal HCO_3^- secretion. Under these experiments, the pH-stat titrations were carried out at a set mucosal pH of 7.800 (typically within ± 0.003 pH units) using 0.01–0.02 mM HCl as titrant and the titration data (pH, volume of titrant added, time elapsed) was sampled to a PC every 10 s. Bicarbonate flux was calculated over 10-min periods (60 data points) using linear regression yielding r^2 values higher than 0.97 in all cases.

2.7. Luminal pH-sensitivity

The in vivo experiments suggested that reduced luminal pH (as set by HEPES buffered salines) resulted in elevated intestinal HCO_3^- secretion. In order to investigate whether this apparent pH-sensitivity observed in vivo could be attributed to local events occurring in isolated epithelia, HCO_3^- secretion was measured at a range of luminal pH-set points. Each intestinal preparation was exposed to luminal pH values in random order ranging from 7.4 to 8.2. The pH-stat titrations at a given set-point were continued until four subsequent 10-min titration periods revealed constant HCO_3^- secretion values. These experiments were conducted with HCO_3^-/CO_2 buffered serosal saline gassed with 0.5% CO_2 in O_2 while titration and data collection were performed as outlined above.

2.8. Analytical techniques

Blood samples (500 μl) were taken through caudal artery/vein catheters into gas-tight 1-ml syringes (Hamilton) and this volume was immediately replaced with heparinised saline. Plasma was obtained by centrifugation of blood for 3 min at 4 °C and $10{,}000 \times g$ (Ole Dich, 157MP). Rectal fluid and blood plasma pH was determined using a Cameron E301 glass electrode in association with E351 reference electrode and a pH/blood gas meter (Cameron BGM200) maintained at the experimental temperatures (13 ± 1 °C). Total CO_2 of rectal fluid or plasma was analysed on 50-μl samples using a carbon dioxide analyser (Mettler Toledo 965). Osmolality of plasma and rectal fluid was measured by vapour pressure osmometry (Wescor, Vapro™). Cations were analysed by atomic absorption spectrometry (Pye Unicam SP9) following appropriate dilution and addition of 0.1% (w/v) $LaCl_3$ for divalent ions. Anions were measured by ion chromatography (Dionex DX-120), except for plasma chloride in the non-cannulated flounder, which was measured using an automatic chloride titrator (Corning 925).

The rectal fluid and carbonate precipitates collected from the rectal catheter bags were initially separated by centrifugation (3 min at 5000 rpm and 4 °C; Sorvall RC 5B Plus, Buch & Holm A/S). Rectal fluids were then decanted off and analysed for pH and total TCO_2 as described above, counted for ^{45}Ca activity and diluted accordingly for ion concentration measurements. The remaining pellet from each fish containing the precipitated carbonates was then rinsed twice in 15 ml of deionized water (Nanopure), re-centrifuging each time, and finally homogenised (Wheaton, Philadelphia, USA, no. 5842) in 10 ml of deionized water for analysis of the bicarbonate equivalent content ($[HCO_3^- + CO_3^{2-}]$) by the double titration method (see Ref. [9]). This involved acidifying each sample to below pH 4.0 with 1.0 N HCl which solubilises all the calcium and magnesium carbonates. At this point, a 1-ml sample was taken for analysis of ion content and ^{45}Ca activity within the mucus.

All water samples from the flux chambers were immediately analysed for pH and then a 25-ml aliquot stored at 4 °C for analysis of titratable alkalinity (T_{Alk}; i.e. bicarbonate

equivalent content) within 24 h as described in Ref. [19]. A second aliquot was stored at −20 °C for later analysis of total ammonia by a colourimetric method [20].

^{45}Ca radioactivity was determined using a TRI-CARB 2500 TR liquid Scintillation analyzer using UltimaGold LSC scintillation fluor (Packard).

2.9. Calculations

Net fluxes between the fish and the external water were calculated using the following equation:

$$J_X^{\text{net}} = [([X]_i - [X]_f)*V]/(M*t)$$

where V is the volume of water (l) in the chamber (after the initial sample was taken), M is the mass of the fish (kg), t is the duration of the flux period (h), and $[X]_i$ and $[X]_f$ are the ion concentrations in the chamber water (μmol l^{-1}) at the beginning and end of the flux period, respectively. Titratable acid flux rates (J_{TA}) were calculated from the above equation using titratable alkalinity measurements (essentially $[HCO_3^- + 2\ CO_3^{2-}]$), but reversing the initial and final values to achieve acid instead of base fluxes. Net acidic equivalent (H^+) flux (J_{H+}^{net}) was determined as the sum, signs considered, of J_{TA} and ammonia excretion (J_{Amm}) as described in Ref. [19]. It should be noted that the net acid flux (J_{H+}^{net}) can result from the movement of any of the following: H^+, NH_4^+, HCO_3^- or OH^-. While it is not possible to distinguish between these forms, H^+ and NH_4^+ excretion, and HCO_3^- and OH^- uptake are all equivalent in terms of the acid–base status of the fish. For all fluxes a negative sign represents a net loss of acid by the animal and positive represents a net gain of acid.

Blood plasma and rectal fluid PCO_2, and bicarbonate and carbonate concentrations were calculated from the measured TCO_2 and pH values according to the Henderson–Hasselbalch equation. For rectal fluid bicarbonate and carbonate concentrations, an apparent second dissociation constant (pK_a) of 9.46 was obtained empirically by direct comparison of the double titration method (see above) with TCO_2 and pH measurements on the rectal fluids from the same fish [9]. For calculating plasma PCO_2 and bicarbonate concentration, dissociation constants and solubility values were used according to Boutilier et al. [21].

Rectal fluid flow rates were calculated from the mass of the rectal fluid within catheter bags at the end of the experimental period and adjusted for the time interval and mass of fish. The rate of flux of ions into or out of intestinal perfusates was calculated as follows:

$$J_X^{\text{net}} = \frac{([X]_{\text{Perf}}) - ([[X]_{\text{RF}} + [X]_{\text{Ppt}})}{(M \times t)}$$

where $[X]_{Perf}$ represents the cumulative quantity of the ion in question infused via the perfusate during the perfusion period, and $[X]_{RF}$ and $[X]_{Ppt}$ represent the total quantity of the ion in question present in the rectal fluid and precipitates collected in the rectal catheter bag over the same period. M is the fish mass (kg) and t is the exact time period (h) over which the animals were perfused. Positive values represent net absorption, negative represent net secretion. The amount of ^{45}Ca, non-radioactive calcium and magnesium that was infused within perfusates and then recovered in either the mucus precipitates, or the rectal fluid + precipitates combined, was calculated by comparing the total amounts collected as a percentage of the total amounts infused. The amounts of ^{45}Ca appearing in the external water during the 3-day fluxes and in the blood and urine upon terminal sampling were used to estimate the percentage of perfusate ^{45}Ca appearing in the extracellular fluid and potentially excreted via the urine. These estimates were based on the following assumptions: (a) ^{45}Ca activity was equal in blood and all extracellular fluids, (b) an extracellular fluid volume (ECFV) of 25–30% of body mass [22,23], (c) ^{45}Ca activity in urine was the same after 3 days as at the start, and (d) a urine flow rate of 0.4 ml kg^{-1} h^{-1} for seawater flatfish [12]. These are all assumptions that will tend to maximise the estimates of % ^{45}Ca recovery in ECFV and urine.

2.10. Statistical analysis

Data are expressed as mean ± S.E. ANOVA was employed to assess significant differences amongst treatments from the in vivo experiments, followed by Bonferroni's test for post hoc pairwise comparisons. For the pH-stat experiments, however, the tests were performed as paired tests. In all cases, $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. In vivo flounder intestinal perfusion experiments—acid–base consequences

As reported previously [9], fish with intestines perfused with 20 mM instead of 5 mM calcium (controls) exhibited a 57% increase in the excretion of total bicarbonate equivalents by the intestine, and a sevenfold increase in the proportion of this attributable to precipitated carbonate (Fig. 1A). Perfusion with HEPES-buffered saline did not affect the overall bicarbonate excretion rate, but halved the proportion that was excreted as carbonate precipitates (Fig. 1A). The rectal fluids of flounder perfused with control and HEPES buffered salines were essentially identical in terms of acid base parameters whereas the pH was reduced by 0.3 units and fluid $[HCO_3^- + CO_3^{2-}]$ was more than halved in the flounder perfused with the high calcium saline (see Table 3). The mean calcium concentrations within rectal fluid were fairly constant (2.4–3.6 mM) regardless of the fourfold difference in concentration within the original perfusate (Fig. 2).

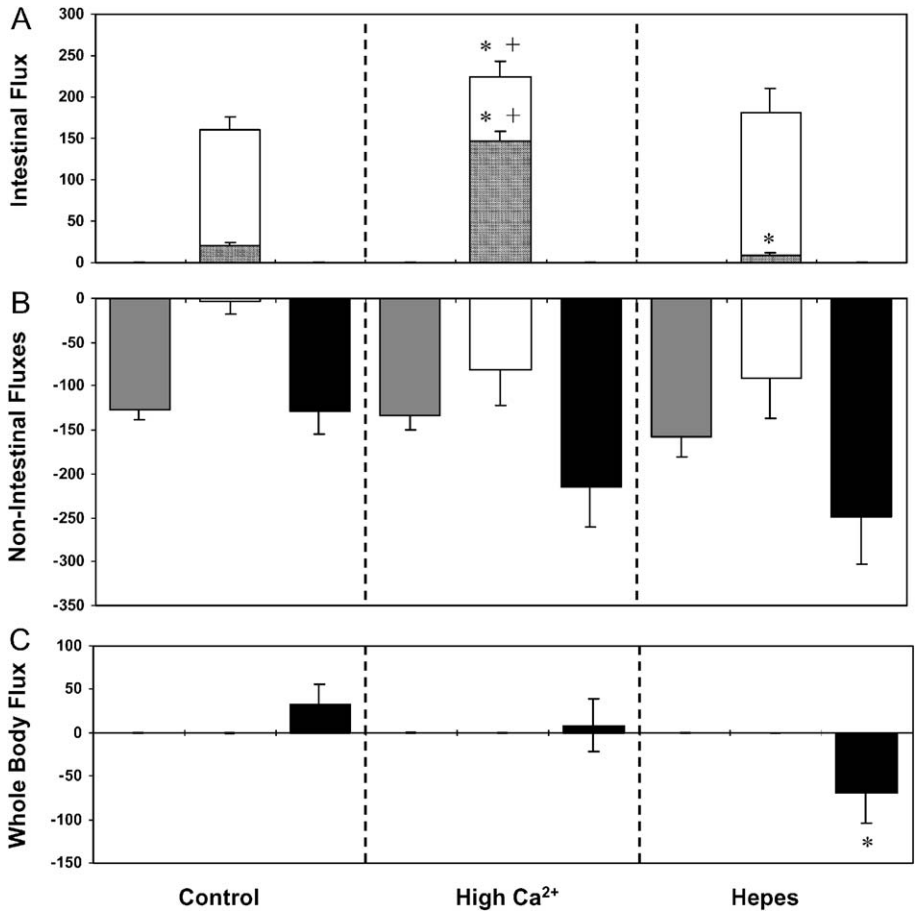

Fig. 1. The net excretion of acid–base equivalents via the intestine (panel A), via non-intestinal routes (panel B; i.e. the gills, kidney and skin), and both these components combined (panel C; i.e. the whole body) in flounder with intestines perfused with one of the three experimental salines (control, high calcium or HEPES buffer—see Section 2 for details of salines) over a 72-h period. Bars represent mean values ± S.E. for seven fish in each treatment. Open bars in panels A and B represent the net titratable acid flux (J_{TA}), which is the reverse of net bicarbonate equivalents flux (i.e. positive represents net base excretion or acid uptake). The hatched bars in panel A represent the proportion of this J_{TA} flux contributed by precipitated carbonates via the intestine. Grey bars in panel B represent the net ammonia flux (J_{Amm}), and black bars in panels B and C represent the net flux of acidic equivalents (J_{H+}^{net}; i.e. the sum of J_{TA} and J_{Amm}, signs considered). Asterisks represent a significant difference from the corresponding control group value, and crosses represent a significant difference from the HEPES buffer group ($P<0.05$).

Regarding the non-intestinal fluxes (i.e. branchial, renal, and skin), there was essentially zero net excretion (or uptake) of bicarbonate equivalents in the control group such that the net acid flux was equivalent to the ammonia excretion rate (Fig. 1B). However, the intestinal bicarbon-

Table 3
pH and calculated [$HCO_3^- + CO_3^{2-}$] concentration of rectal fluids collected from flounder in the three intestinal perfusion treatments

	pH	[$HCO_3^- + 2CO_3^{2-}$] (mEq l^{-1})
Controls	8.72 ± 0.05	67.8 ± 9.2
High Ca^{2+}	8.41 ± 0.06**	33.7 ± 3.5**
HEPES	8.73 ± 0.02	65.8 ± 8.1

Values are means ± SE, $n=6$. Double asterisk indicates significantly different from both control and HEPES buffer treatments at $P<0.001$. These data have already been published (in Ref. [9]) and are repeated here for completeness.

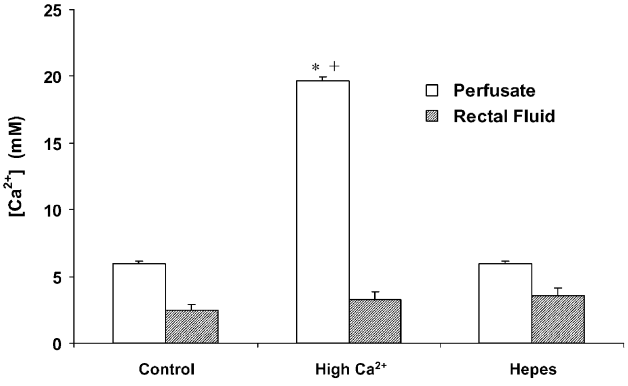

Fig. 2. Mean values (± S.E.) for the calcium concentration entering (perfusates) and leaving the intestine (rectal fluid) from flounder perfused with either control, high calcium, or HEPES buffered salines. Asterisks represent a significant difference from the corresponding control group value, and crosses represent a significant difference from the HEPES buffer group ($P<0.05$).

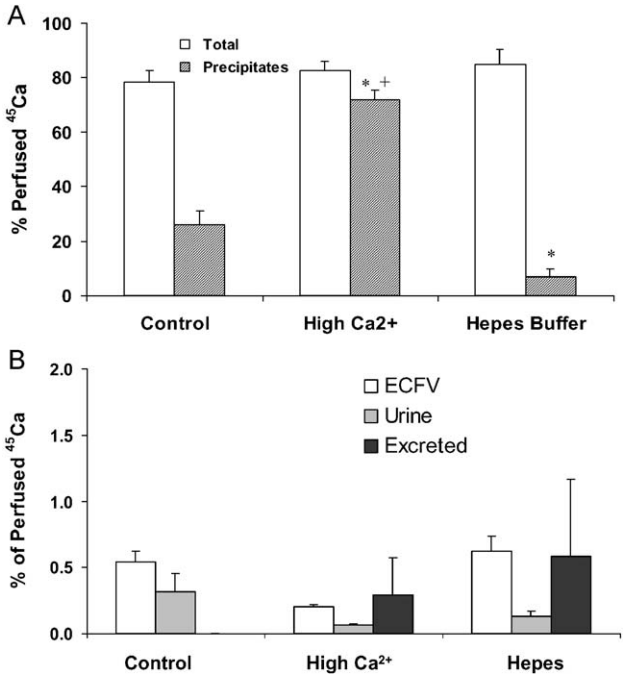

Fig. 3. The appearance (recovery) of ^{45}Ca in different compartments expressed as a percentage of the total quantity of ^{45}Ca originally entering the intestine within the perfusates. Panel A shows the total amount of ^{45}Ca that passes through the intestine unabsorbed (i.e. within the combined rectal fluid and excreted precipitates—open bars) as well as the proportion associated solely with the carbonate precipitates (grey bars). Panel B shows estimates (see Section 2 for details) of the amount of ^{45}Ca appearing in the extracellular fluid volume (ECFV—open bars) and excreted over the 3-day period via the urine (grey bars), as well as the measured quantity of ^{45}Ca actually excreted into the external seawater (by any route) over the 3-day period. Asterisks represent a significant difference from the corresponding control group value, and crosses represent a significant difference from the HEPES buffer group ($P<0.05$).

ate excretion rate (Fig. 1A) was slightly higher than the whole animal ammonia excretion rate (Fig. 1B), resulting in a small net acid uptake for the whole animal (i.e. positive J_{H+}^{net}; or net excretion of base overall). In the high Ca^{2+}-perfusion group, the additional bicarbonate excreted by the intestine (Fig. 1A) was approximately matched by an apparent net uptake of bicarbonate (or acid excretion) via non-intestinal routes (i.e. negative J_{TA}^{net}; Fig. 1B). As the ammonia excretion rate was unaffected, the whole animal net acid flux remained similar to that of the control group (Fig. 1C). In the flounder perfused with a HEPES-buffered saline, ammonia excretion was unaffected but there was an apparent net uptake of bicarbonate via non-intestinal routes (similar to the high-Ca^{2+} treatment but in the absence of a stimulation of intestinal net bicarbonate excretion). Thus, there was a net excretion of acid equivalents from the whole animal in this group, which was significantly different from the small net acid uptake observed in the control group (Fig. 1C). However, there was no significant effect of any perfusion treatment on blood acid–base status when measured after 60 h; mean blood pH in the three treatments ranged from 7.94 ± 0.03 to 7.97 ± 0.03, mean plasma $[HCO_3^-]$ values ranged from 6.90 ± 0.38 to 7.48 ± 0.61 mM, and the mean PCO_2 values ranged from 1.78 ± 0.15 to 1.84 ± 0.12 mm Hg.

3.2. *In vivo flounder intestinal perfusion experiments—consequences for divalent ion balance*

The proportion of ^{45}Ca passing unabsorbed through the intestine was very constant at around 80% (Fig. 3), regardless of the initial calcium concentration in perfusates or the buffering of perfusate that limited the amount of $CaCO_3$ precipitation (Fig. 1A). A similar picture can be seen from the measurements of total (non-radioactive) calcium, although the estimates are much more variable (Table 4) with means ranging from 60% to 94% unabsorbed Ca^{2+}. Measurements of total magnesium revealed a more consistent picture, with about 90% passing through the intestine without being absorbed in all treatments. A very small proportion of the perfused magnesium (<4%) ended up in precipitated mucus carbonates. This contrasts with calcium where as much as 75% of the perfused calcium (measured as either ^{45}Ca or total calcium) was present in the excreted carbonate precipitates in the group perfused with high-Ca^{2+} saline (significantly higher than in the control and HEPES groups). Even in the control group, almost 40% of the total perfusate calcium was recovered within the precipitated carbonates. This was reduced to 10% by the addition of HEPES buffer to the perfusion saline (Table 4), similar to the reduction in precipitation of carbonate (Fig. 1A).

The amount of calcium recovered in the rectal fluid excretions suggests that up to 20% of the calcium perfused through the intestine might be absorbed (Fig. 3, Table 4). However, Fig. 4 shows that only 0.5% to 1.5% of the ^{45}Ca in perfusates could be accounted for by the combined measurements of ^{45}Ca appearing in the extracellular fluid, urine, and external water. In addition, there were no significant differences in the concentration of calcium in plasma after 60 h of each treatment (mean values in the three treatments ranged from 3.01 ± 0.20 to 3.23 ± 0.11 mM, $N=7$).

Table 4
The amount of calcium and magnesium present in the original intestinal perfusates subsequently recovered from contents of the rectal catheter bags

		% Recovery of perfused Ca^{2+}	% Recovery of perfused Mg^{2+}
Controls	Total	71.4 ± 3.9	90.1 ± 2.0
	Mucus	(37.9 ± 8.6)	(1.6 ± 0.7)
High Ca^{2+}	Total	94.4 ± 7.3 +	88.3 ± 4.6
	Mucus	(76.8 ± 7.9)*+	(3.9 ± 0.5)
HEPES	Total	59.5 ± 10.2	91.6 ± 1.5
	Mucus	(10.8 ± 4.9)	(0.4 ± 0.3)

Values are shown as mean % recoveries ± S.E., for the total recovery (in both rectal fluid and precipitated mucus). Values in parentheses represent the amounts recovered from the precipitated mucus alone.

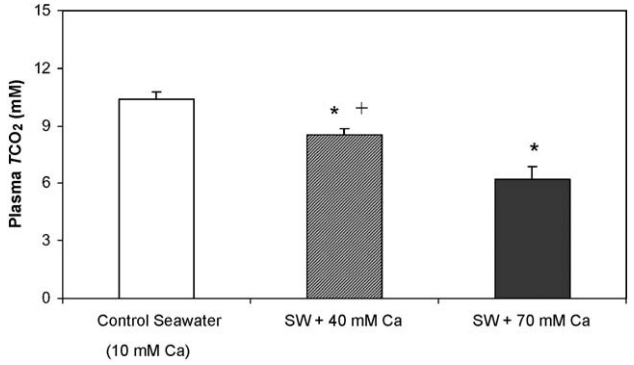

Fig. 4. The plasma total CO_2 concentration in flounder exposed to variable ambient calcium concentrations (10, 40 or 70 mM) in natural seawater. Calcium was added as $CaCl_2$ and the osmolality of seawater was adjusted with deionised water to that for control seawater (see Section 2 for details).

3.3. In vivo exposure of flounder to seawater with elevated calcium concentrations

Increasing the ambient calcium concentration from 10 to 40 and 70 mM caused progressive and significant reductions in the plasma total CO_2 concentration in flounder acclimated to these conditions for 3 days (Fig. 4). At the highest ambient calcium concentration, plasma TCO_2 was reduced by 40%. Plasma calcium and chloride concentrations were not significantly affected by additional ambient calcium but plasma osmolality was significantly reduced from 320 ± 1 mosM kg^{-1} in the control seawater group to 310 ± 1 mosM kg^{-1} at the highest ambient $[Ca^{2+}]$ of 70 mM.

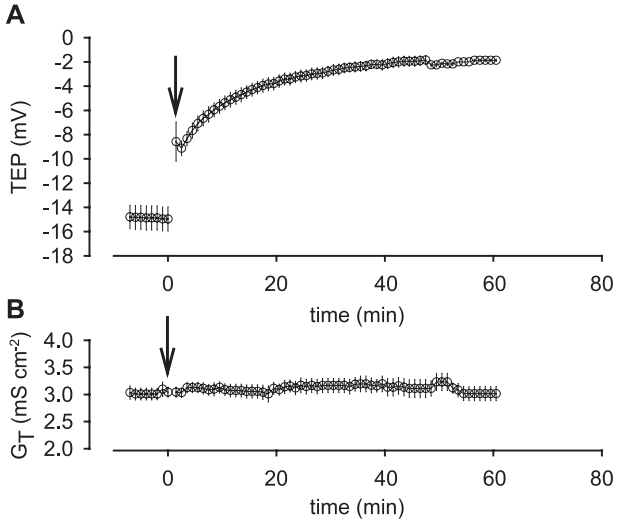

Fig. 5. Transepithelial potential (TEP) (A) and conductance (GT) (B) of the anterior intestinal epithelium from the European flounder under asymmetrical in vivo-like conditions and under symmetrical conditions. Arrows indicate time of change from asymmetrical to symmetrical conditions. Mean ± S.E. ($n = 6$).

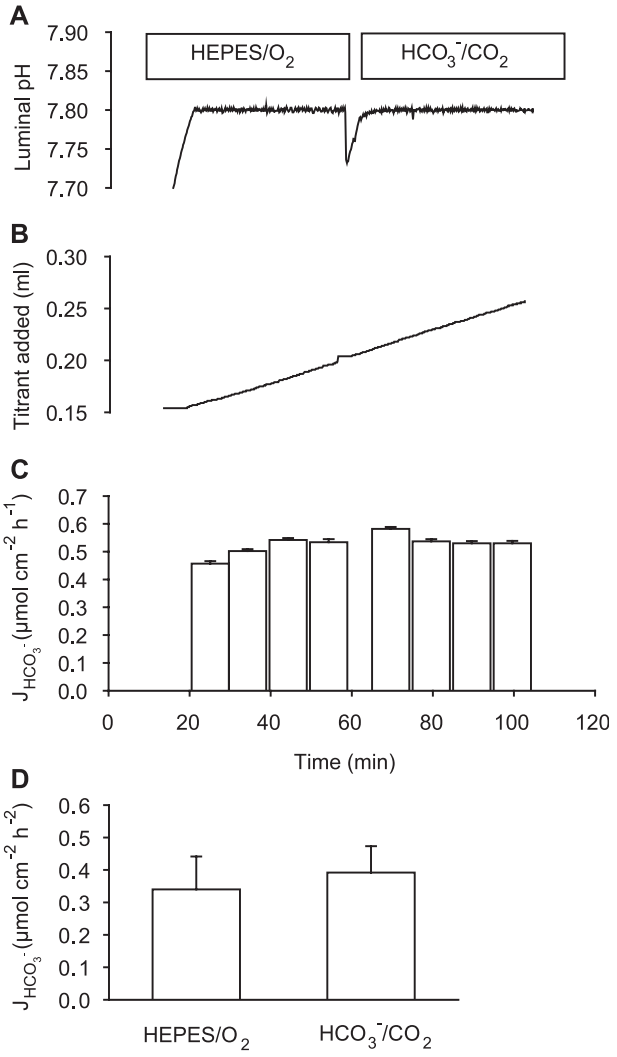

Fig. 6. Panels A–C show an illustrative example of data obtained during a pH-stat experiment including recorded pH during a 100-min experiment (A), volume of titrant added over time to maintain constant pH (B) and finally the calculated epithelial HCO_3^- secretion (C). Panel D shows the combined results of six experiments performed on isolated anterior intestine from European flounder as mean ± S.E.

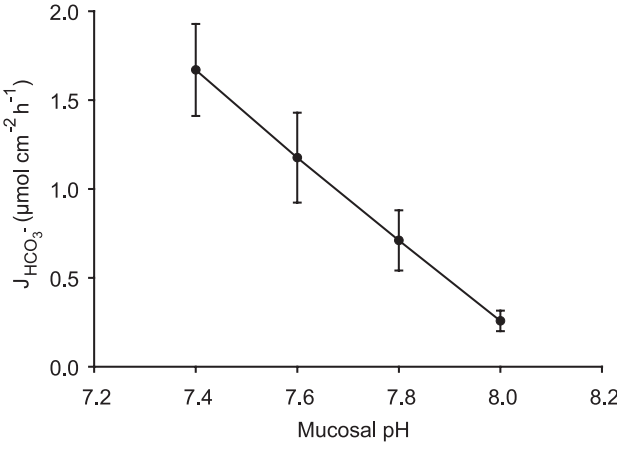

Fig. 7. Apparent HCO_3^- secretion by isolated anterior intestine of European flounder as a function of luminal pH. Mean ± S.E. ($n = 3$).

Table 5
Transepithelial potential (TEP) and conductance (G) (mean ± S.E.) across the anterior intestine of the European flounder exposed to different luminal pH ($n=3$)

pH	TEP (mV)	G (mSi cm^{-2})
7.4	− 14.5 ± 0.5	2.50 ± 0.13
7.6	− 14.0 ± 1.0	2.46 ± 0.16
7.8	− 13.5 ± 1.5	2.66 ± 0.36
8.0	− 14.5 ± 0.5	2.46 ± 0.16

3.4. In vitro pH-stat system

The mean TEP under asymmetrical condition was − 15 mV serosal-side negative but gradually dropped to − 2 mV over 60 min after exposure to symmetrical conditions (Fig. 5A). Mean epithelial conductance under asymmetrical conditions was 3 mS cm^{-2} and remained constant at this value after change to symmetrical conditions (Fig. 5B).

An illustrative example of data obtained during a pH stat experiment is shown in Fig. 6A–C. Fig. 6A shows pH 7.800 maintained during a pH-stat experiment while Fig. 6B shows the volume of titrant added over time. The corresponding calculated HCO_3^- secretion values in subsequent 10-mín titration periods prior to and after replacement of the serosal HEPES/O_2 saline with the HCO_3^-/CO_2 saline are shown in Fig. 6C. Mean values from experiments on six individual preparations revealed no effect of serosal presence of HCO_3^-/CO_2 on HCO_3^- secretion (Fig. 6D). Transepithelial potential and epithelial conductance were not influenced by the presence or absence of serosal HCO_3^-/CO_2.

Bicarbonate secretion in isolated epithelia was dependent on luminal pH exhibiting higher apparent secretion rates at lower pH values (Fig. 7); however, luminal pH did not influence electrophysiological parameters (Table 5). Maximal luminal alkalinization was between 8.0 and 8.2 as evident from the y-axis intercept on Fig. 7. None of the tested preparations showed HCO_3^- secretion at pH 8.2.

4. Discussion

4.1. Acid–base consequences of intestinal HCO_3^- secretion and sources of HCO_3^-

The up-regulation of intestinal HCO_3^- secretion in response to elevated luminal calcium (Fig. 1A) corresponds to an enhanced net excretion of base via the intestine. For acid–base balance to be maintained, there must be an equivalent excretion of acid (or base uptake) elsewhere in the body. This must have occurred as blood acid–base status was unperturbed after 60 h of perfusion with high calcium saline, and the whole animal net acidic equivalent flux was the same as the control group over the 3-day period. A compensatory acid excretion/base uptake most likely occurred via the gills, as the kidney in marine teleosts has a very limited capacity for acid–base relevant transfers due to the very low flow rates [14,15] even though urine acid content tends to be slightly higher in seawater-adapted than freshwater teleosts [12,15]. When compared with the control group, the non-intestinal base uptake (negative J_{TA} in Fig. 1B) was enhanced (though not quite significantly compared to the control group; $P=0.057$) by almost exactly the same degree as the enhanced intestinal base excretion (Fig. 1A). Extra HCO_3^- uptake (or its acid–base equivalent, e.g. proton extrusion) via the gills is the most likely source of the compensatory acid–base flux during stimulation of intestinal HCO_3^- excretion with additional calcium.

Thus, it appears that the intestine has the potential to up-regulate base secretion (as HCO_3^-) when required for additional removal of calcium (by carbonate precipitation), and that, within limits (e.g. ~ 60% increase), long-term acid–base disturbances are avoided by a similar net uptake of HCO_3^- equivalents (or excretion of H^+) elsewhere, likely the gills, with no net effect on blood acid–base parameters. However, when pushed further, such compensatory systems do not seem to maintain blood acid–base status constant. In the in vivo perfusion study, flounder intestines were exposed to 15 mM extra calcium compared to controls. In the in vivo study using non-cannulated flounder, the ambient seawater was supplemented with an additional 30 and 60 mM calcium over and above the control level of 10 mM. These elevations caused significant and 'dose'-dependent decreases in plasma total CO_2 concentration (Fig. 4). Blood pH was not measured in this experiment, so a complete picture of acid–base status is not available. Assuming that these higher calcium concentrations reach the intestinal lumen and proportionally stimulate intestinal bicarbonate secretion, it would appear that at some point the additional excretion of HCO_3^- exceeds the supply from endogenous hydration of CO_2 within the intestinal epithelium, and starts to draw upon the circulating store of CO_2/HCO_3^-, ultimately reducing plasma total CO_2. This scenario complements in vitro data from the flounder intestine. For example, Fig. 6 shows that serosal CO_2 is not required for baseline HCO_3^- secretion in the intestine, indicating that endogenous hydration of CO_2 is sufficient to supply HCO_3^- for apical exchange with chloride under normal circumstances. At the same time, raising serosal CO_2 from 0.5% to 2% in vitro can apparently stimulate the HCO_3^- secretion rate (Grosell et al., unpublished results). Thus, it would appear that at high enough Ca^{2+} levels, HCO_3^- secretion can be up-regulated to the point where the endogenous supply of CO_2 is insufficient and exogenous CO_2 stores start being utilised resulting in reduced blood total CO_2 content. Conversely, artificially raised blood CO_2 availability can drive up the apical HCO_3^- secretion rate.

4.2. Role of intestinal HCO_3^- secretion in divalent cation homeostasis

When comparing the dissolved calcium concentration in imbibed seawater (~ 10 mM) with that in the excreted

rectal fluid (~ 3 mM) and considering that 50–80% of the imbibed water volume is absorbed during transit through the intestine, it is perhaps not surprising that many authors have described this organ as the site of a large net calcium uptake in marine teleosts [3,11,13]. However, this analysis ignores the suggestion originally made by Shehadeh and Gordon in 1969 [2], that when the excretion of precipitated carbonates is taken into account, the proportion of imbibed calcium absorbed by the intestine may actually be rather low. Under control conditions, in the present study, less than 20% of the calcium entering the intestine appears to be absorbed with 30–40% being excreted as precipitated $CaCO_3$, the remainder as dissolved Ca^{2+} in rectal fluid. Although the precipitates do contain a relatively high content of magnesium carbonate, the contribution this makes to net magnesium excretion is rather low (<4%). This is because the dissolved Mg^{2+} concentrations are much higher than calcium in seawater (and the intestinal salines used in the present study), and net magnesium absorption is even lower than for calcium, with >90% being recovered in rectal fluid. The more intriguing result is that when the intestine was presented with artificially elevated calcium concentrations, the rate of HCO_3^- secretion appears to up-regulate to the point where virtually all the 'extra' calcium can be accounted for by the increased formation and excretion of $CaCO_3$ precipitates (Fig. 3; Table 4). Thus, the rate of HCO_3^- secretion seems to be well matched to the need for precipitating calcium. This indicates an important role in the homeostasis of this divalent cation, specifically in minimising the potential for excessive calcium entry. The effectiveness of this system for removing imbibed calcium is further demonstrated by the constancy of plasma Ca^{2+} concentration when flounder were exposed to seawater supplemented with seven times the normal ambient Ca^{2+} concentration.

To our knowledge, this is the first study in which ^{45}Ca has been used to track the precise fate of calcium absorbed by the intestine in marine fish. The mass balance for ^{45}Ca entering and leaving the intestine suggested that slightly less than 20% is unaccounted for and presumably absorbed into the bloodstream. However, with the present data, it is difficult to confidently conclude that this is the case. If 20% of the radiolabelled calcium entering the intestine was absorbed over the 3-day period, then one might expect this to show up as substantial ^{45}Ca counts in the blood, urine and external water (if excreted either by the kidney or gills). However, even very liberal estimates of urine flow rates, equilibration times, etc. produce recovery values that account for only 1% of the perfused ^{45}Ca at best. Indeed, under control conditions, ^{45}Ca could not be detected at all in the external water in any of the seven fish over the 3-day period. This could indicate two problems. First, we did not measure ^{45}Ca activity in any tissues that might sequester calcium. This might be particularly true of bone, which contains the vast majority of the whole body calcium pool in teleost fish [24] and could therefore act as a very significant reservoir for radiolabelled ^{45}Ca entering the animal. However, this would depend on the rate of exchange of free calcium in circulation with the mineral salts (apatites) present in bone (predominantly calcium phosphate and calcium carbonate; [24]). Alternatively, the intestinal tissue itself could provide an important reservoir of our unaccounted for ^{45}Ca. Little is known about the mechanism and rate of formation of mucus and incorporation of carbonate precipitates by the intestinal epithelium [25]. In the present study, we only collected the precipitates that had already separated from the intestinal wall or been voided from the intestine. If the process takes hours rather than minutes, then a significant proportion of the perfused calcium (and ^{45}Ca) could have remained unabsorbed but incorporated into the newly formed mucus coating the intestinal epithelium, even after 3 days of perfusion. Thus, 20% is probably an upper limit for the absorption of calcium from imbibed seawater by the intestine. Indeed the percent recovery of total calcium (rather than ^{45}Ca) from the high Ca^{2+} perfusate was not significantly different from 100% (Table 4). Although it is hard to explain the differential estimates based on the ^{45}Ca and total Ca^{2+} data, it raises the question of how little (if any) calcium might be absorbed under different conditions. Clearly, further measurements of different body pools are required to resolve the missing fraction of calcium passing along the intestine of marine fish.

4.3. pH sensitivity of HCO_3^- secretion

During the in vivo study in which flounder were perfused with intestinal saline buffered with 5 mM HEPES, the net HCO_3^- secretion via the intestine was not affected, but there was a significant stimulation of net acid excretion by the whole animal. The 5 mM HEPES buffer would effectively clamp the luminal pH to ~ 7.4 by providing a source of free protons within the intestinal lumen. Thus, initially at least, much of the secreted HCO_3^- would be converted to CO_2 by the high availability of these free protons, and the elevated CO_2 would ultimately diffuse into the blood with the potential for causing an acidosis. This would explain the stimulation of whole animal net acid excretion. Indeed, based on in vitro titrations of our 5 mM HEPES saline using NaOH, and the perfusion rate of the HEPES saline, it would have required the intestinal base excretion (or acid absorption) rate to be 78 μmol kg^{-1} h^{-1} higher than in the control group to achieve the rectal fluid pH actually observed in these two groups (~ 8.7, Table 3). This is almost identical to the additional whole animal net acid excretion observed in the HEPES group, which indicates that acid–base homeostasis was achieved by excreting (probably via the gills) the additional protons gained from titrating the intestinal supply of HEPES buffer.

Presumably the additional base excretion required to titrate the 5 mM HEPES came from secreted HCO_3^-, but this was subsequently converted to CO_2 and hence would not contribute to the net intestinal HCO_3^- excretion rate seen in Fig. 1A. If this interpretation is correct then it suggests

that HCO_3^- secretion is stimulated by keeping the lumen pH lower (using the HEPES buffer) at least at the anterior section of the intestine. This pH sensitivity is supported by the in vitro data shown in Fig. 7, where the HCO_3^- secretion rate increased linearly as mucosal pH was reduced from 8.2 to 7.4. From the in vivo experiment, it is impossible to determine whether HCO_3^- secretion might be stimulated simply due to an enhanced transepithelial HCO_3^- gradient at lower pH, or due to a direct effect of pH on the secretory process. The in vitro pH-stat experiments offer more insight into this phenomenon. It is possible that reducing mucosal pH simply reduces the luminal bicarbonate concentration (by shifting CO_2/HCO_3^- equilibrium to the left) and thus promoting the gradient for further HCO_3^- secretion across the apical membrane. However, this is not a likely explanation for the pH-stat observations because the system operates to continuously titrate mucosal HCO_3^- to CO_2 that is subsequently gassed off by equilibration with CO_2-free O_2. Thus, the mucosal HCO_3^- concentration should be constant and negligible regardless of the HCO_3^- secretion rates seen during these in vitro experiments. Constant gassing and simultaneous stirring of both the two half-chambers also acted to minimise the presence of any unstirred layers. In addition, varying luminal pH did not influence the epithelial potential and thus did not alter the electrical component of the driving force for any transepithelial HCO_3^- transport (Table 5). This all supports the idea that luminal pH affects HCO_3^- secretion by a mechanism other than altering the local HCO_3^- gradient. Our observations of apparent pH-stimulated HCO_3^- secretion in response to luminal acidification is in close agreement with the fact that luminal acidification is the main stimulator of mammalian duodenal HCO_3^- secretion [26–29]. The similarity to mammalian systems is logical given that imbibed seawater entering the anterior intestine will have been at least partially acidified during passage through the stomach (at least in those fish possessing a true gastric region; [30]). Low pH in the luminal fluid should therefore signal the entrance of fluid via the stomach and help initiate HCO_3^- secretion (and perhaps other ion transport processes thought to be important in water absorption?).

This pH sensitivity would presumably work in conjunction with the stimulatory effect of high calcium concentrations also present in the imbibed seawater (Ref. [9] and present study). We have proposed that the stimulatory effect of calcium is likely to be via Ca^{2+}-sensing receptors (CaRs) in the apical membrane of intestinal epithelial cells [9]. This is supported by the findings of Nearing et al. [31] who recently proposed that CaR might act as a salinity sensor generally in fish, and found it to be expressed within a variety of tissues, including the apical membrane of the intestinal epithelium in teleosts. In contrast, we do not have a proposed mechanism for how luminal pH is sensed and regulates intestinal HCO_3^- secretion. In mammals the mechanism by which duodenal HCO_3^- secretion is stimulated by low pH is not fully understood although speculation has included H^+-sensitive neural receptors or cell filaments within the apical mucus layer [29]. Alternatively, due to the effective barrier that mucus provides for H^+ ions, it has even been postulated that the epithelium might sense the more rapidly diffusable CO_2, generated from reaction of secreted HCO_3^- with gastric H^+, rather than H^+ itself [32]. For marine teleosts, the mechanism for pH sensitivity clearly remains to be investigated. In addition, it will be of interest to establish how Ca^{2+} and H^+ might interact in their stimulatory action in marine teleost fish, as both would be physiologically relevant agonists of intestinal HCO_3^- secretion in association with drinking and ultimately osmoregulation.

5. Conclusions

In conclusion, HCO_3^- secretion in the intestine of marine teleosts is stimulated by both Ca^{2+} and low pH. Under normal circumstances, the secreted HCO_3^- base seems to originate from the endogenous hydration of CO_2 within epithelial cells, and an equalising quantity of acid secretion occurs elsewhere, likely at the gills. At higher rates of HCO_3^- secretion, stimulated by elevated Ca^{2+}, exogenous (plasma) CO_2 becomes an important source of HCO_3^-, and extracellular acid–base status can be subsequently affected. The HCO_3^- secretion serves to assist in both water absorption and calcium homeostasis by precipitating calcium as carbonates and thereby reducing luminal fluid osmolality and minimising calcium absorption. The latter is likely to be an important mechanism in defending the kidney from renal stone formation as marine teleosts live in a hypercalcaemic environment that only allows minimal urine flow rates and prevents the use of dilution to excrete salts with poor solubility. Further research should be directed at investigating the mechanisms underlying the sensing of luminal Ca^{2+} and pH and their subsequent regulation of HCO_3^- secretion in particular with respect to osmotic and ionic regulation.

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