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Review

Intestinal bicarbonate secretion by marine teleost fish—why and how?

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Abstract

Intestinal fluids of most marine teleosts are alkaline (pH 8.4–9.0) and contain high levels of HCO_3^- equivalents (40–130 mM) which are excreted at a significant rate (>100 μEq kg^{-1} h^{-1}). Recent research reveals the following about this substantial HCO_3^- secretion: (1) It is not involved in acid–base regulation or neutralisation of stomach acid, but increases in parallel with drinking rate at elevated ambient salinities suggesting a role in osmoregulation; (2) In species examined so far, all sections of the intestine can secrete bicarbonate; (3) The secretion is dependent on mucosal Cl^-, sensitive to mucosal DIDS, and immuno-histochemistry indicates involvement of an apical Cl^-/HCO_3^- exchanger. In addition, hydration of CO_2 via carbonic anhydrase in combination with proton extrusion appears to be essential for bicarbonate secretion. The mode of proton extrusion is currently unknown but potential mechanisms are discussed. One consequence of the luminal alkalinity and high bicarbonate concentrations is precipitation of calcium and magnesium as carbonate complexes. This precipitation is hypothesised to reduce the osmolality of intestinal fluids and thus play a potential role in water absorption and osmoregulation. The present studies on European flounder reveal that elevated luminal calcium (but not magnesium) concentrations stimulate intestinal bicarbonate secretion both acutely and chronically, in vitro and in vivo. At the whole animal level, the result of this elevated bicarbonate secretion was increased calcium precipitation with an associated reduction in the osmolality of rectal fluids and plasma. These observations suggest direct functional links between intestinal bicarbonate secretion, divalent cation precipitation and osmoregulation in marine teleost fish.
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Keywords: Calcium; Precipitation; Osmoregulation; pH-stat titration; Water absorption; Chloride–bicarbonate exchange

1. Introduction

Marine teleost fish are hypo-osmotic to the medium they inhabit. To avoid dehydration, they drink the external seawater, absorb the majority of the imbibed volume within the intestine, and the remainder is excreted as rectal fluid [1–3]. However, it was only in 1991 that Walsh et al. [4] reported that the fluid within the intestinal and rectal fluids are both alkaline (pH 8.4–9.0) and rich in the basic HCO_3^- and CO_3^{2-} ions (40–130 mM; see also Ref. [5]). This is well above electrochemical equilibrium with extracellular fluids [6,7], with typical plasma [HCO_3^-] values of 6–10 mM in marine teleosts [8]. This has since been confirmed for a variety (14) of marine teleosts [6,7,9,10]. Somewhat earlier, Shehedah and Gordon [2] had recognised (but not actually measured) that the rectal fluids of seawater rainbow trout most probably contained high concentrations of bicarbonate. This supposition was based on their discovery of carbonate precipitates within the intestinal mucus 'tubes' they found within these fish. The presence of such carbonate precipitates has also now been documented in virtually all the marine teleosts investigated for this purpose [6,7].

Two important questions arise when considering this alkaline intestinal environment and the precipitation of carbonate salts that it produces. First, what is the primary function of producing such a bicarbonate-rich environment, and second, what are the mechanisms by which the epithelium delivers such high concentrations to the intestinal lumen? In other words, why and how? The importance of these questions to understanding piscine adaptations to the marine environment is clear when one realises that virtually all marine teleosts appear to produce a bicarbonate-rich intestinal fluid. Presumably the physiological processes involved must have appeared early in the evolutionary history of teleosts that can inhabit seawater.

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1.1. Why?

This rather unusual intestinal environment is far more alkaline than that of mammals [11] and occurs even when marine teleosts are deprived of food. This indicates that the function of the intestinal bicarbonate is not simply associated with digestion via the neutralisation of gastric acid [9]. In addition, the intestinal epithelium itself appears to be the major source of the secreted bicarbonate in marine teleosts [7,9,10,12], departing from the standard mammalian scenario of a bicarbonate-rich secretion from the pancreas that serves to just match and neutralise the acidic chyme entering from the stomach (duodenal contents rarely exceed pH 6–7; [11]).

Due to their habit of almost continuously drinking seawater and voiding the unabsorbed rectal fluid, the excretion of bicarbonate equivalents via this route is sufficient to make a significant contribution to the acid–base balance of marine teleosts (generally >100 μEq kg^{-1} h^{-1} in seawater-acclimated teleosts; [6,9]). This gave rise to the proposal that the intestine may play a role in acid–base regulation, a previously unexplored route for controlling the excretion of acid–base relevant ions. However, subsequent studies on both the euryhaline rainbow trout (*Onchorhynchus mykiss*), and the stenohaline flathead sole (*Hippoglossoides elassodon*) demonstrated that the rate of HCO_3^- base excretion via the intestine was essentially insensitive to disturbances in systemic acid–base status, for example to $NaHCO_3$ loading and resultant metabolic alkalosis (Wilson, R.W., Tierney, M.L. and Wood, C.M., unpublished data). Thus, while it is a quantitatively important component of resting acid–base fluxes with the environment, bicarbonate excretion via the intestine does not appear to be dynamically regulated for the control of systemic acid–base status per se.

1.2. Role in osmoregulation—divalent cation precipitation

The presence of this phenomenon in marine teleosts and its absence in their freshwater counterparts suggests it may be related to the osmoregulatory strategy of fish living in a hyper-osmotic environment [4,6]. Furthermore, there is a linear relationship between the intestinal HCO_3^- excretion rate and external salinity in both trout and flounder (Wilson, R.W., Tierney, M.L., Bury, N.R., Hogstrand, C., Wood, C.M., Grosell, M., Jensen, F.B., Lecklin, T., Busk, M., unpublished data) once the salinity becomes hyper-osmotic to the body fluids (above ~ 10 ppt). Given the high content of calcium and magnesium in the carbonate precipitates, it is interesting to speculate that bicarbonate secretion may facilitate the osmoregulatory function of the intestine in seawater-adapted teleosts by precipitating these divalent ions as their insoluble carbonates. A negative correlation between intestinal fluid Ca^{2+} and $HCO_3^- + CO_3^{2-}$ concentrations (Fig. 1) certainly indicates that intestinal bicarbonate secretion affects luminal Ca^{2+} presumably through precipitation. This would reduce the effective osmolality of fluid along the intestine (because the precipitates exert no osmotic pressure) and thereby promote further water absorption. This idea is expressed in the model presented in Fig. 2, which uses data from rainbow trout acclimated to seawater to represent the theoretical

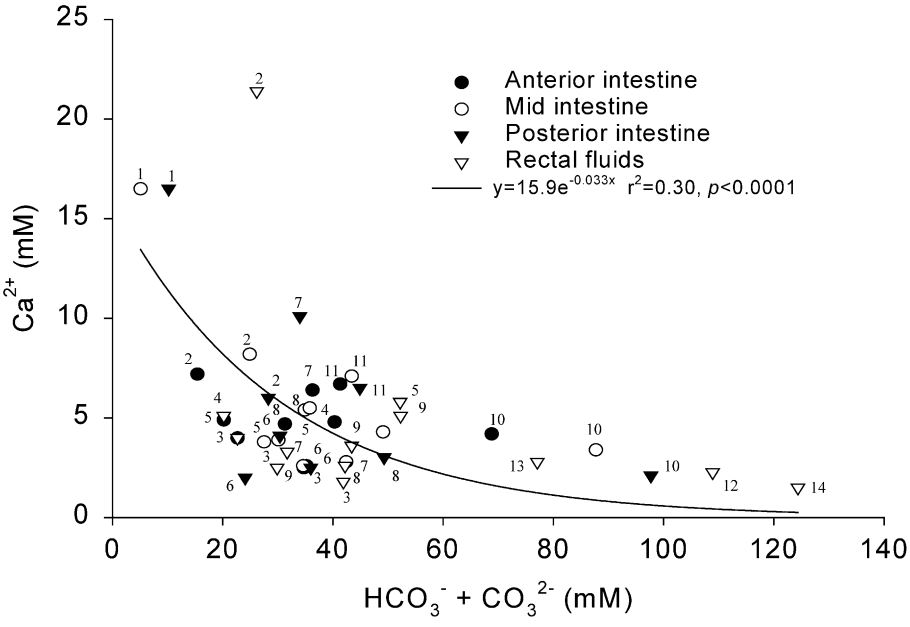

Fig. 1. Correlation between intestinal $HCO_3^- + CO_3^{2-}$ and total Ca^{2+} concentrations in intestinal fluids from 14 marine teleosts. Original data from Refs. [6,7]. Numbers on the figure refer to the following species: 1, *Gadus morhua*; 2, *Zoarces viviparous*; 3, *Parophrys vetulus*; 4, *Limand limanda*; 5, *Scophthamus maximus*; 6, *Cyclopterus lumpus*; 7, *Myoxocephalus scorpius*; 8, *Pleuronectes platessa*; 9, *Scophthalmus rhombus*; 10, *Plactichtys flesus*; 11, *Citharichthys sordidus*; 12, *Oncorhynchus mykiss*; 13, *Hippoglosoides elassodon*; 14, *Anguilla anguilla*. For additional information see Refs. [6,7].

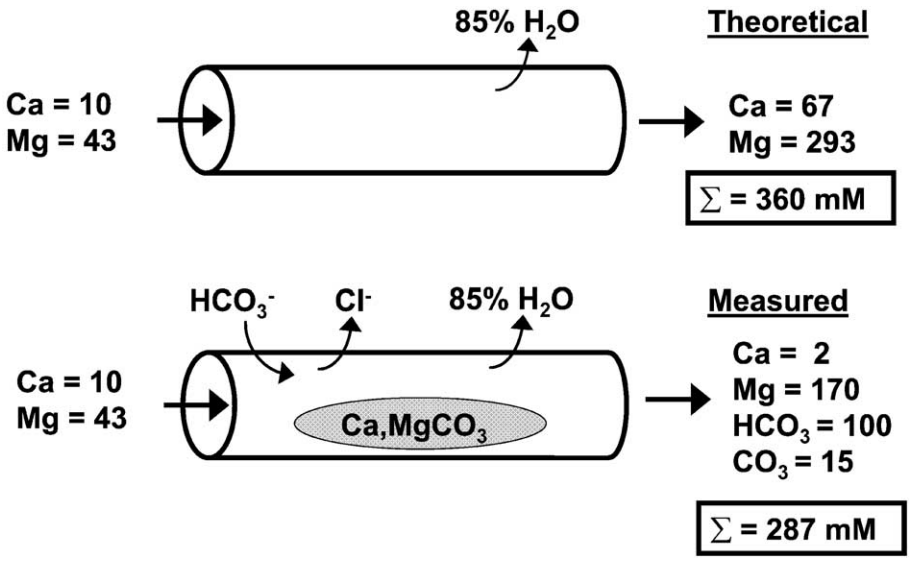

Fig. 2. A model representing the theoretical effects of intestinal HCO_3^- secretion on the osmolality of luminal fluids by causing the precipitation of imbibed calcium and magnesium as carbonates. Numbers represent concentrations of ions in mM, and only the ions in question are considered (Ca^{2+}, Mg^{2+}, HCO_3^-, CO_3^{2-}). The intestine is treated as a simple tube with normal 35-ppt sea water being imbibed on the left hand side. The upper scenario represents the predicted concentrations of Ca^{2+} and Mg^{2+} in the rectal fluid if normal rates of water reabsorption occur (~ 85%; [9]), and assumes no Ca^{2+} or Mg^{2+} is absorbed by the intestine, and both remain in solution. The lower scenario shows the actual measured concentrations of Ca^{2+} and Mg^{2+} in the rectal fluid of seawater-acclimated rainbow trout [9] in which the intestine secretes HCO_3^-, resulting in a final concentration of ~ 115 mM of $HCO_3^- + CO_3^{2-}$ with the precipitation of 97% and 42% of the theoretical dissolved Ca^{2+} and Mg^{2+}. This represents a difference in total concentrations of these ions of 73 mM. The associated lowering of osmolality would allow considerably more water to be reabsorbed by the intestine.

effects of HCO_3^- secretion on the osmolality of intestinal fluids by causing the precipitation of calcium and magnesium carbonates. While this simplified model assumes zero absorption of calcium and magnesium (which is undoubtedly incorrect to a small extent; [2,13]), it does demonstrate a theoretical lowering of the luminal concentrations of these ions by 73 mM. It could be argued that the impact would actually be greater than this because the model in Fig. 2 does not consider that the HCO_3^- is secreted in exchange for chloride which is subsequently removed from the luminal fluid. Whatever the actual figure, the lowering of luminal ion concentrations is due to the precipitation of divalent cations that would otherwise become concentrated to extremely high levels and hence restrict the absorption of water from the intestinal lumen. In water reabsorbing epithelia such as the mammalian kidney proximal tubule, the localised (intra-epithelial) osmotic gradient set up by active transport to drive substantial net water absorption is thought to be in the range of 5–6 mOsm/kg [14,15]. Given the magnitude of this gradient, a reduction in luminal osmolality due to 73 mM fewer ions in solution (Fig. 2) would afford a considerable advantage in terms of facilitating further reabsorption. Nevertheless, although this theoretical analysis suggests a potentially important role in osmoregulation, experiments have not yet been attempted to test this hypothesis.

1.3. Role in osmoregulation—Cl^- absorption

Clear correlation between bicarbonate secretion and chloride absorption [9] and luminal chloride and HCO_3^- concentrations in the lumen of a high number of teleost species (Fig. 3) points to a role of the bicarbonate secretion system in chloride absorption. Generally, salt and water absorption across marine teleost intestines has been attributed to Na^+:Cl^- and Na^+:K^+:$2Cl^-$ co-transporters [16], but Cl^-/HCO_3^- exchange now seems to contribute significantly to intestinal chloride absorption. In the Pacific sand dab, removal of luminal chloride completely abolished sodium and water absorption whereas some chloride and water absorption persisted under sodium free conditions [7]. This demonstrates that sodium-independent chloride uptake pathways exist in the intestinal epithelium and that these pathways can drive water absorption. Studies employing in situ intestinal perfusion on the lemon sole revealed that net chloride absorption was double that of net sodium absorption [10]. The net bicarbonate secretion matched the difference between chloride and sodium absorption, suggesting that a chloride–bicarbonate exchange system contributes significantly to chloride absorption in this marine teleost. That this phenomenon appears to be general is illustrated by Fig. 3, which shows a strong correlation between luminal Cl^- and $HCO_3^- + CO_3^{2-}$ concentrations in intestinal fluids obtained from a large number of marine teleost species. Although the stochiometry of the anion exchanger is normally 1 chloride ion for 1 bicarbonate ion, if bicarbonate is derived from cellular CO_2 (which as a gas has no osmotic influence), then the exchange will effectively result in a net movement of osmolytes into the cell from the lumen of the intestine, and hence will help drive some water absorption. The subsequent removal of any secreted bicarbonate as

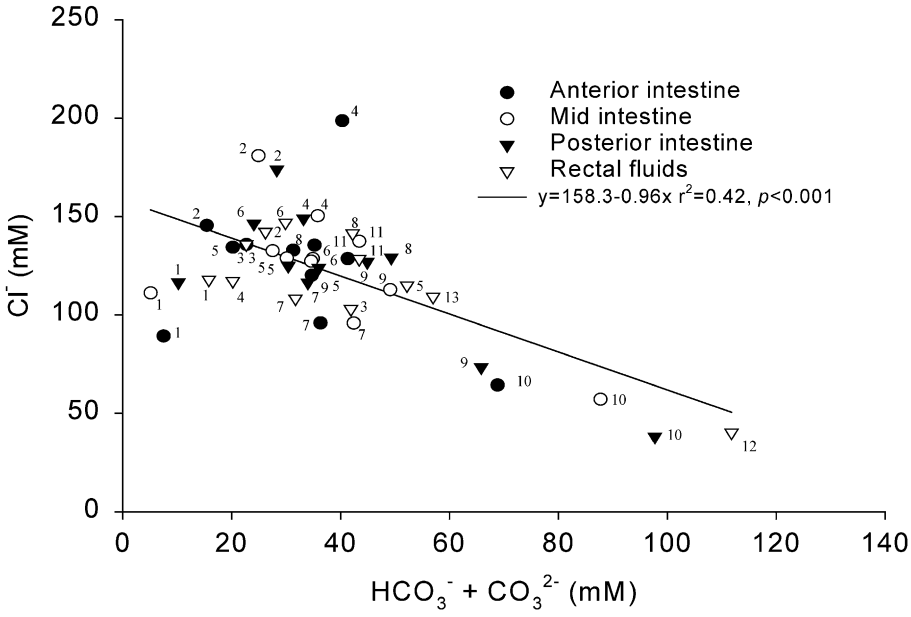

Fig. 3. Correlation between intestinal $HCO_3^- + CO_3^{2-}$ and Cl^- concentrations in intestinal fluids from 12 marine teleosts. Original data from Refs. [6,7]. 1, *Gadus morhua*; 2, *Zoarces viviparous*; 3, *Parophrys vetulus*; 4, *Limand limanda*; 5, *Scophthamus maximus*; 6, *Cyclopterus lumpus*; 7, *Myoxocephalus scorpius*; 8, *Pleuronectes platessa*; 9, *Scophthalmus rhombus*; 10, *Platichtys flesus*; 11, *Citharichthys sordidus*; 12, *Oncorhynchus mykiss*; For additional information see Refs. [6,7].

precipitated carbonate will obviously further this advantageous osmotic effect.

1.4. How?—Mechanism of bicarbonate secretion

Active secretion is necessary to account for the substantial bicarbonate gradient across the leaky epithelial barrier between the intestinal lumen and the extracellular fluid in marine teleost fish. The potential across the intestinal epithelium mounted in Ussing type chambers under symmetrical conditions is typically less than − 2 mV (blood side negative; see Ref. [16] for a review). Although potentials reported from preparations exposed to in vivo like (asymmetrical) conditions are typically higher, e.g. − 3 to − 8 mV [17,18]), they cannot account for the luminal bicarbonate concentrations in most teleost fish [4,6,7,9,10,19] which are often 10-fold higher than the extracellular bicarbonate concentrations.

The luminal bicarbonate concentration is high even in the most anterior part of the intestine of most teleosts, suggesting that the majority of the intestinal bicarbonate secretion occurs in this region. However, comparing bicarbonate secretion by isolated intestinal segments from three different marine teleosts reveals that the mid and posterior regions also exhibit substantial bicarbonate secretion (Fig. 4). This suggests that

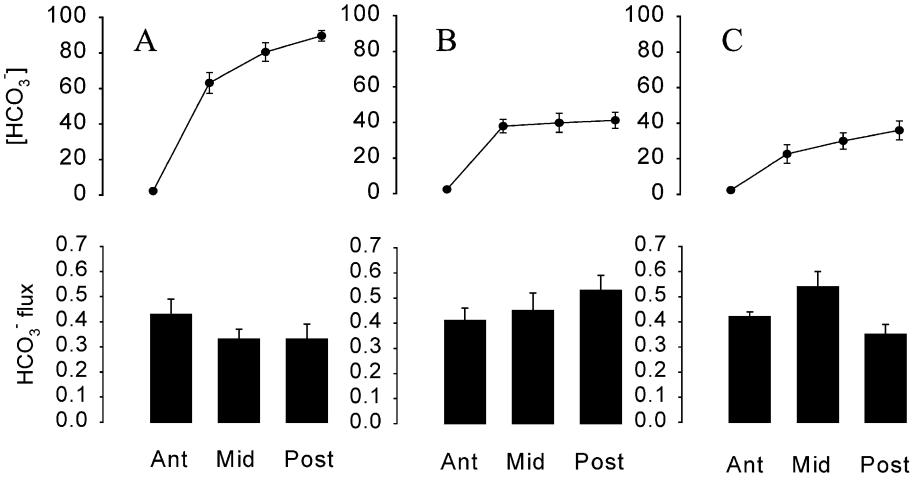

Fig. 4. Luminal bicarbonate concentration (top panels) in the anterior, mid and posterior region of the intestine and bicarbonate secretion by corresponding isolated anterior, mid and posterior segments (bottom panels) under in vivo like conditions from European flounder (A), Pacific Sanddab (B) and lemon sole (C). Mean values ± S.E. See Refs. [7,10,19] for further details.

while all regions of the intestine have the capacity to secrete bicarbonate, this does not necessarily occur in vivo, presumably due to differences in luminal chemistry along the intestine. Current models [16] fail to explain how the intestinal epithelium secretes bicarbonate against an electrochemical gradient and include only a basolateral Cl^-/HCO_3^- anion exchanger (AE). This localization of the AE is based on original observations of reduced bicarbonate secretion after serosal addition of DIDS [20]. Since then, however, several reports of reduced bicarbonate secretion after mucosal application of DIDS have appeared [7,19,21], suggesting an apical localization of an intestinal AE. Additional evidence for apical localization include strong correlation between intestinal chloride absorption and bicarbonate secretion in seawater adapted rainbow trout [9] and chloride-dependent bicarbonate secretion by isolated intestinal segments [7]. Perhaps the most compelling evidence in support of an apical localization of Cl^-/HCO_3^- exchanger in the marine teleost intestine is immunohistological observations clearly demonstrating cross-reactivity of an AE1 antibody with the apical membrane in two different marine/euryhaline teleosts (Figs. 5a and 6c). Interestingly, seawater-acclimated coho salmon exhibit clear apical localization of Cl^-/HCO_3^- exchangers, whereas acclimation to freshwater eliminates this polarization (Fig. 5). The latter again supports the idea that intestinal bicarbonate secretion plays a role in marine teleost osmoregulation (see above).

An anion exchanger in itself is not sufficient to explain apparent active bicarbonate transport, indicating the involvement of other components to intestinal bicarbonate secretion. Extracellular fluid bicarbonate and CO_2 as well as endogenous CO_2 arising from epithelial-cell respiration are all potential sources of bicarbonate for luminal secretion. Carbonic anhydrase-mediated hydration of CO_2 yielding bicarbonate and protons is probably involved in the process since application of acetazolamide (a specific carbonic anhydrase inhibitor) reduces bicarbonate secretion [9,21]. The protons generated from CO_2 hydration must be extruded from the epithelial cells in order to prevent a reversal of the catalysed CO_2 to HCO_3^- reaction and thus maintain sufficient cytosolic bicarbonate to drive apical secretion. Furthermore, the proton extrusion must occur across the basolateral membrane since the intestinal epithelium exhibits strong net base secretion [7]. Proton extrusion across the basolateral membrane could be either via a proton pump as seen in human pancreatic ducts [22] or via a sodium–proton exchanger as seen in the rat pancreatic ducts [23]. Transepithelial bicarbonate secretion could also be mediated by a basolateral Na^+:HCO_3^- co-transporter providing cytosolic bicarbonate for the apical anion exchange process as documented in the guinea-pig pancreatic ducts [24]. If the source of bicarbonate is CO_2 hydration, either the proton pump directly (through the extrusion of protons) or the sodium–potassium pump (indirecty) could provide the energy for active bicarbonate secretion. In the latter case, the Na^+/K^+ ATPase establishes the sodium gradient required for Na^+/H^+ exchange via the Na^+/H^+ exchanger and could thus fuel proton extrusion indirectly. The sodium gradient established by Na^+/K^+ ATPase could also fuel bicarbonate secretion by providing energy for sodium–bicarbonate co-transport. Evidence for involvement of a sodium-dependent transporter in bicarbonate

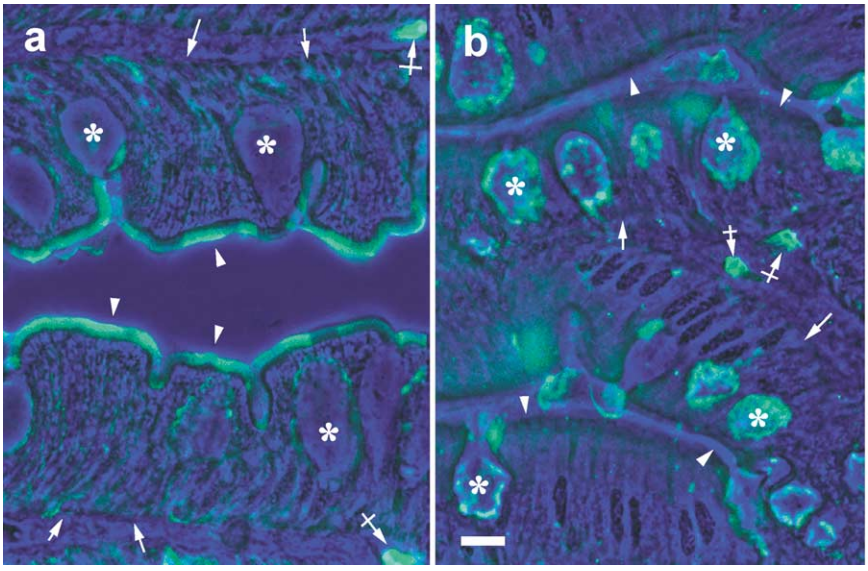

Fig. 5. Immunofluorescence localization of a band 3-like anion exchanger (AE1) protein in the intestinal villi of seawater (a) and freshwater (b) acclimated coho salmon (*Oncorhynchus kisutch*). Sections were indirectly labelled with a rabbit polyclonal anti-trout band 3 (AE1t) and an FITC-conjugated secondary antibody. The corresponding phase-contrast images are shown in overlay in blue. Arrowheads and arrows indicate the enterocyte brush boarder and basolateral membranes, respectively. Asterisks indicate goblet cells and crossed-arrows indicate erythrocytes. Tissue was fixed in Bouin's solution and embedded in paraffin. Sections were pretreated with SDS prior to immunolabelling (see Ref. [34]). Scale bar = 25 μm.

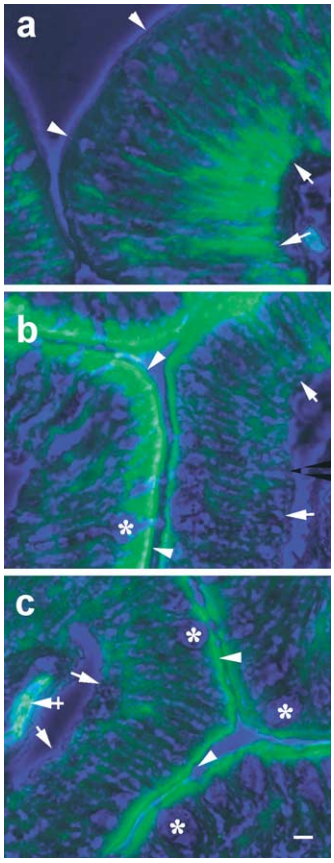

Fig. 6. Localization of basolateral (a) Na^+/K^+-ATPase and apical (b) $Na^+:K^+:2Cl^-$ co-transporter and (c) band 3-like protein in the intestinal epithelium of the brackish water mudskipper (*Periophthalmodon schlosseri*) by immunofluorescence using antibodies α5, T4 and AE1t, respectively (see Refs. [34,35]). Corresponding phase-contrast images are shown in overlay in blue. See Fig. 1 for label descriptions. Scale bar = 10 μm.

secretion was first reported by Ando and Subramanyam [21] for marine Japanese eel showing reduced bicarbonate secretion under sodium free conditions. This has also been documented for the lemon sole [7]. It is however unclear whether this sodium dependence is due to Na^+/H^+ exchange, $Na^+:HCO_3^-$ co-transport or a sodium dependant Cl^-/HCO_3^- exchanger as reported from mouse pancreatic islets [25].

1.5. Aims

One aim of the present study was to determine the role of bicarbonate secretion in calcium precipitation within the intestinal fluid, and the subsequent influence this has on water absorption and whole animal osmoregulation. If bicarbonate secretion is important for precipitation of divalent ions, one could hypothesise that the rate of secretion might be proportional to the amount of calcium entering the intestine. Thus, elevated luminal calcium concentrations should stimulate intestinal bicarbonate secretion and result in greater calcium carbonate precipitation. Conversely, buffering the fluid entering the intestine to a more neutral pH should reduce the formation of CO_3^{2-} ions and inhibit calcium carbonate precipitation. In whole animal experiments, to negate the complicating influence of ion and osmotic exchanges at other sites (e.g. the gills), an in vivo intestinal perfusion technique was adopted [7,10,19]. Using this method the medium entering the intestine could be selectively altered in the different treatments, with the gills of all fish being bathed by the same external salinity and ion concentrations. Since bicarbonate secretion responded as predicted to increased calcium in intestinal perfusates in situ, the presence of a similar response in isolated intestinal segments was tested using an in vitro pH-stat approach.

2. Materials and methods

2.1. Animal source and holding

European flounder (*Platichthys flesus*; 150–1500 g) were obtained from a commercial fishing boat fishing in the northern part of the Sound of Copenhagen Denmark and held in a recirculating seawater system at 30 ppt salinity and 13 ± 1 °C. The artificial seawater was made by adding sea salt (Instant Ocean) to Copenhagen city tap water. Recirculating seawater (total volume of 4000 l) was passed through a protein skimmer (6000 l h^{-1}) and a biological filter to ensure optimal water chemistry. Each holding tank contained fine gravel covering a bottom filter driven by an airlift. Fish were not fed after capture to eliminate the contamination of intestinal fluids with faecal matter.

2.2. In vivo 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 [26] 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 [7,10,19]. Stomach drain catheters and intestinal perfusion catheters where fashioned from 3–5-cm lengths of polyethylene tubing (Portex; ID = 1.02 mm OD = 1.98 mm) heat-flared at the end to be inserted. A 2-cm incision was made in the uppermost surface of the abdominal cavity running perpendicular to the body length and beginning just ventral to the pectoral fin. The junction between the stomach and intestine was exposed and externalised via this incision (located by visualising the two small pyloric caecae just distal to the pyloric sphincter). A small incision was made just anterior to the pyloric sphincter. The stomach drain catheter was inserted via this incision towards the oesophagus, and the intestinal perfusion catheter was inserted via the same incision and gently pushed through the pyloric sphincter.

Both catheters were then secured in place with sutures tied around the stomach wall and the incision in the body wall closed with sutures. The intestine was then flushed via the perfusion catheter with 20 ml of the saline to be used during the experiment. During flushing the abdomen was massaged to ensure removal of all the endogenous intestinal fluid and mucus precipitates. Each fish was then fitted with a rectal catheter bag fashioned from a condom with the open end tied tightly around a 1-cm length of ridged plastic tubing (ID ~ 5 mm, OD ~ 7 mm) which was inserted into the anus and held in place by a purse-string ligature. Incisions for blood and intestinal catheters 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 seawater 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 36 and 60 h of the perfusion.

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 precipitation of calcium by bicarbonate secretion influences intestinal water absorption and whole body osmoregulation. The control saline (see Table 1) contained 5 mM Ca^{2+} similar to the level found in vivo in rectal fluid. The second group were perfused with a high calcium saline (20 mM) in an attempt to enhance precipitation and the potential for a reduction in luminal osmolality of rectal fluid. The third saline contained the normal calcium concentration (5 mM) but had 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 precipitation of $CaCO_3$ by reducing the potential for alkalinisation and formation of CO_3^{2-} ions caused by intestinal bicarbonate secretion. This in turn was expected to minimise the osmotic advantage of eliminating divalent ions from solution as carbonates. See Table 1 for a detailed description of the three salines used. All salines were exactly matched with respect to osmolality by the addition of an appropriate amount of mannitol prior to use in perfusions. In addition, 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. At the end of experiments flounder were exanguinated within the flux chambers and the perfusion catheter carefully detached from the peristaltic pump. Fish were then removed from the flux chamber and rinsed in freshwater 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.

Table 1
Nominal composition of salines used for intestinal perfusion

	Control	High Ca	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 7.5)	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 vitro procedures—pH-stat experiments

In order to investigate whether the calcium-stimulation of bicarbonate secretion observed in vivo could be attributed to local events occurring in isolated epithelia, a pH-stat approach was employed. 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 a 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 (0.01–0.02 N HCl) via an autoburette (ABU 901, Radiometer) coupled to an automatic pH-stat controller (PHM290, Radiometer). 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). 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 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

Table 2
Composition of salines used in pH-stat experiments

	Serosal	Mucosal
Na^+	145.9	50
Mg^{2+}	1.5	100
Ca^{2+}	6.0	5
K^+	2.5	5
Cl^-	139.5	110
SO_4^{2-}	1.5	77.5
PO_4^{3-}	3.0	
HCO_3^-	11.9	
Osmolality	283	281.5

The pH-stat set point used was pH 7.8. Concentrations of inorganic ions are in mM, and unit of osmolality is mOsm kg^{-1}.

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.

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 N HCl as titrant and the titration data (pH, volume of titrant added, time elapsed) were 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. Four subsequent 10-min periods exhibiting constant flux values was employed as the criteria for stable control conditions prior to experimental manipulations. The latter consisted of adding stock solutions of $CaCl_2$ or $MgCl_2$ to the mucosal bath to increase the concentration of these divalent ions by 5 mM. Preparations were followed for a minimum of 60 min after addition of these salts.

In order to compare bicarbonate flux rates obtained from the pH-stat experiments with whole animal base secretion, the total gross surface area of the intestine from the pyloric to the rectal sphincter was determined from European flounder (176–474 g, $n=4$) using graph paper.

2.5. 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 heparinsed saline. Plasma was obtained by centrifugation of blood for 3 min at 4 °C and 10,000 × *g* (Ole Dich, 157MP). Rectal fluid pH was determined using a Cameron E301 glass electrode in association with E351 reference electrode and a pH/blood gas meter (Cameron BGM200). All electrodes were thermostatically controlled to the experimental temperatures (13 ± 1 °C). Total CO_2 of rectal fluid was analysed on 50-μl samples using a carbon dioxide analyser (Mettler Toledo 965). Osmolarity 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).

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 and Holm A/S). Rectal fluids were then decanted off and analysed for pH and total CO_2 as described above. The remaining mucus 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 [27]. This involved titrating each sample to below pH 4.0 with 1.0 N HCl and titrating back to the starting pH with 0.2 N NaOH. During titrations samples were continuously aerated with CO_2-free air to remove all HCO_3^- and CO_3^{2-} as gaseous CO_2 during acidification, and to ensure stable pH measurement when returning to the starting pH. The differential in the number of moles of HCl and NaOH required to return to the starting pH is then equivalent to the number of moles of $HCO_3^- + CO_3^{2-}$ equivalents in the original sample. For all titrations combination pH electrodes were used (Hanna, HI 1230) in conjunction with a pH meter (either Hanna, HI 8314 or Russell, RL 200). Acid and base were added using 2-ml micrometer syringes (Gilmont Instruments, Barrington, USA).

2.6. Calculations

Rectal fluid bicarbonate and carbonate concentrations were calculated from the measured TCO_2 and pH values according to the Henderson–Hasselbalch equations and using a second dissociation constant (pK_a) of 9.46. This value 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. This is slightly lower than published values of pK_a for seawater of the same temperature and salinity as the flounder rectal fluids ($pK_a = 9.62$; [28]).

The rate of excretion of bicarbonate base equivalents ($HCO_3^- + 2CO_3^{2-}$) was calculated as follows:

$$J_{\text{net}}^{\text{HCO3}-} = \frac{[\text{B}]_{\text{RF}} + [\text{B}]_{\text{PPT}}}{(M \times t)}$$

where $[B]_{RF}$ and $[B]_{PPT}$ represent the total quantity of bicarbonate equivalents ($[HCO_3^- + 2CO_3^{2-}]$ in μequiv) in the rectal fluid and precipitates collected in the rectal catheter bag, M is the fish mass (kg), and t is the time period (h) over which the animals were perfused.

2.7. Statistical analysis

Data are expressed as mean ± S.E. ANOVA was employed to assess significant differences amongst treatments

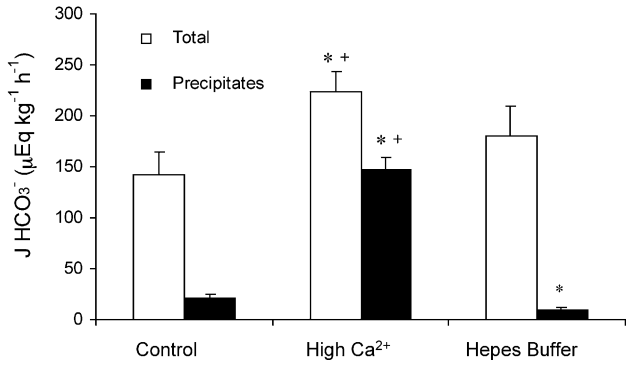

Fig. 7. The net excretion rate of bicarbonate equivalents ($HCO_3^- + CO_3^{2-}$) via the intestine in flounder with intestines perfused with one of the three experimental salines (control, high calcium or Hepes buffer—see Materials and methods for details of salines) over a 72-h period. Bars represent mean values ± S.E. for seven fish in each treatment. The solid bars represent the bicarbonate equivalents excreted as precipitated carbonates. Open bars represent the total bicarbonate equivalents excreted (rectal fluid + precipitates). Asterisks represent a significant difference from the corresponding control group value, and crosses represent a significant difference from the Hepes buffer group ($P < 0.05$).

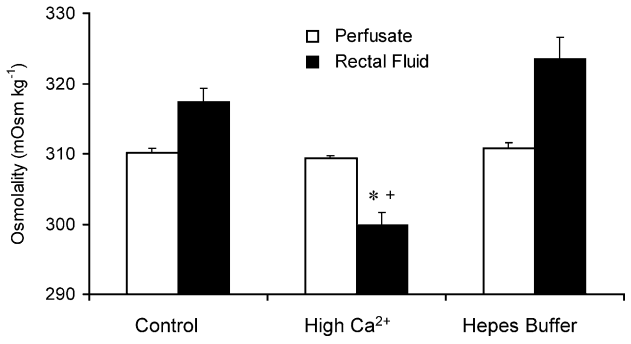

Fig. 8. Osmolality of the perfusate (salines prior to perfusion) and the rectal fluid collected over 72 h from European flounder perfused with one of the three experimental salines (control, high calcium or Hepes buffer—see Materials and methods for details of salines). Bars represent mean values ± S.E. for seven fish in each treatment. The solid bars represent the rectal fluid osmolality and open bars represent the osmolality of the saline prior to perfusion of the intestine. Asterisks represent a significant difference from the corresponding control group value, and crosses represent a significant difference from the Hepes buffer group ($P < 0.05$).

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 *t*-test. In all cases, $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. In vivo experiments

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. 7). Perfusion with Hepes-buffered saline did not affect the overall bicarbonate excretion rate, but halved the proportion that was excreted as carbonate precipitates (Fig. 7). 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 $[HCO_3^- + CO_3^{2-}]$ was more than halved in the flounder perfused with the high calcium saline (Table 3). There were no significant differences in the calcium concentration in rectal fluid of fish from the three treatments (Table 3).

Over the 72-h perfusion period, there were no significant differences between the three treatments with respect to the rate of water absorption by the intestine. The mean values for percent water absorbed from the three perfusates were 38.5 ± 2.1 (6), 42.4 ± 3.0 (7), and 35.9 ± 1.8 (6) in the control, high Ca^{2+} and Hepes groups, respectively. However, there were significant differences in the osmolality of both the collected rectal fluids and the blood plasma, despite the salines having identical osmolalities prior to infusion (Figs. 8 and 9). Osmolalities of the rectal fluid and plasma from the high Ca^{2+} perfusate group were 17 and 7 mOsm kg^{-1} lower than the controls, and 24 and 11 mOsm kg^{-1} lower than in the group perfused with Hepes buffer, respectively.

Table 3
pH, TCO_2, calculated $[HCO_3^- + CO_3^{2-}]$ and calcium concentration of rectal fluids collected from flounder in the three intestinal perfusion treatments

	Control	High Ca	Buffered
pH	8.72 ± 0.05	8.41 ± 0.06**	8.73 ± 0.02
TCO_2 (mM)	58.1 ± 7.5	31.0 ± 3.0**	56.8 ± 6.9
$[HCO_3^- + CO_3^{2-}]$ (mEq/l)	67.8 ± 9.2	33.7 ± 3.5**	65.8 ± 8.1
$[Ca^{2+}]$ (mM)	2.42 ± 0.44	3.28 ± 0.60	3.59 ± 0.60

Values are means ± S.E., $n = 6$. ANOVA was employed to assess significant differences amongst treatments, followed by Bonferroni's test for post hoc pairwise comparisons. Double asterisk indicates significantly different from both control and Hepes buffer treatments at $P < 0.001$.

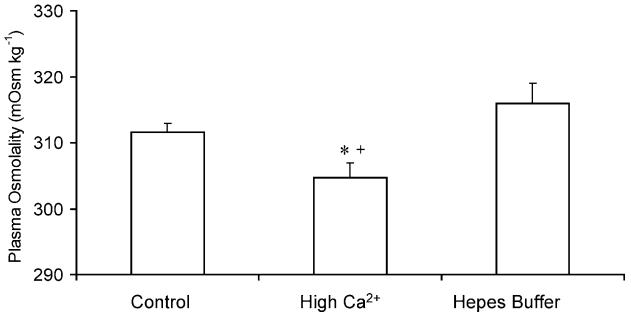

Fig. 9. Osmolality of the plasma of European flounder following 72-h intestinal perfusion with one of the three experimental salines (control, high calcium or Hepes buffer—see Materials and methods for details of salines). Bars represent mean values ± S.E. for seven fish in each treatment. Asterisks represent a significant difference from the corresponding control group value, and crosses represent a significant difference from the Hepes buffer group ($P < 0.05$).

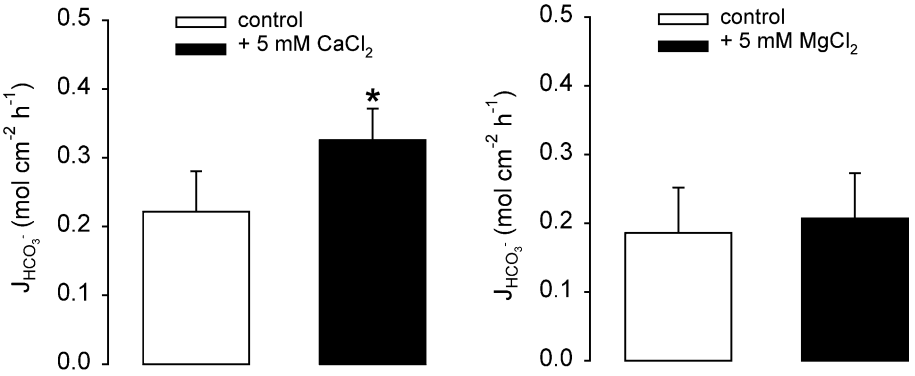

Fig. 10. Bicarbonate flux (μmol cm^{-2} h^{-1}) in isolated anterior intestinal segments from the European flounder prior to (control) and after 5 mM $CaCl_2$ ($n=4$) or $MgCl_2$ ($n=3$) addition. * Denotes statistical significant difference from corresponding control (paired t-test).

3.2. In vitro experiments

The pH-stat experiments on isolated intestinal segments revealed substantial bicarbonate secretion. Using the flux rates obtained from the control experiments and the total gross surface area of intestine in European flounder (136 cm^{-2} kg^{-1}, $n=4$), the observed base secretion rates translates to 49 μmol kg^{-1} h^{-1} compared to 145 μmol kg^{-1} h^{-1} obtained from the in situ perfusion experiments. Although bicarbonate secretion rates by the isolated intestinal segments were lower than the in vivo rates, the isolated intestinal segment still responded to elevated luminal calcium (from 5 to 10 mM; added as $CaCl_2$) by a 47% increase (Fig. 10), not dissimilar to the relative response to high calcium observed in vivo (Fig. 7). Adding 5 mM $MgCl_2$ (giving identical increases in chloride concentration and osmolality) did not influence bicarbonate secretion (Fig. 10). Addition of calcium as calcium gluconate was also attempted and did stimulate bicarbonate secretion (data not shown). It is not possible to make sufficiently concentrated stock solutions of calcium–gluconate to spike the mucosal saline during experiments. Consequently, these experiments involved a complete change of mucosal saline which resulted in unstable base secretion rates for up to an hour after manipulation. Nevertheless, addition of calcium gluconate stimulated bicarbonate secretion to a similar extent as with calcium chloride although with a delayed response. The response to elevated calcium did not include significant changes in electrophysiological properties of the isolated intestine (Table 4).

Table 4
Transepithelial potential (TEP) and conductance (G) (mean ± S.E.) across the anterior intestine of the European flounder prior to and after addition of 5 mM $CaCl_2$ ($n=4$) or $MgCl_2$ ($n=3$)

	TEP (mV)	G (mSi cm^{-2})
Control	− 13.8 ± 1.7	3.17 ± 0.12
+5 mM $CaCl_2$	− 13.0 ± 1.3	3.15 ± 0.05
Control	− 11.1 ± 3.8	3.26 ± 0.09
+5 mM $MgCl_2$	− 9.5 ± 2.6	3.26 ± 0.04

Elevated calcium or magnesium did not cause any statistically significant changes in electrophysiological properties of the isolated intestine.

4. Discussion

4.1. Calcium stimulates intestinal bicarbonate secretion

There is clear evidence from both the in vitro pH-stat and in vivo experiments that elevating mucosal calcium above the normal level of 5 mM stimulates the secretion of bicarbonate by the intestine (Figs. 7 and 10). This seems to be a specific response to calcium, rather than either the accompanying chloride anion, osmolality, or the other major divalent cation imbibed in seawater (magnesium), because (a) addition of $MgCl_2$ had no stimulatory effect in the pH-stat experiments (Fig. 10), (b) chloride content was constant in the in vivo experiments where the additional 15 mM of $CaCl_2$ simply replaced 15 mM $MgCl_2$ in the high calcium saline (Table 1 and Fig. 7), and (c) calcium gluconate addition also stimulated bicarbonate secretion (data not shown).

The in vitro pH-stat system utilises an isolated intestinal segment. This together with the rapid response of J_{HCO3} (within minutes) to mucosal calcium suggests that the stimulatory mechanism is dependent upon local events within the epithelium rather than a systemic endocrine-mediated process. The precise mechanism for this local effect of high mucosal calcium remains to be elucidated but possibilities include some form of apical calcium-sensing receptor (see Ref. [29] for a review), and a transporter-mediated elevation of intracellular calcium that in turn stimulates the bicarbonate secretion. Alternatively, it is possible that precipitation of calcium carbonate simply reduces the luminal bicarbonate/carbonate concentration, thus promoting the gradient for further secretion across the apical membrane. However, this seems unlikely given that the stimulation by calcium was equally apparent using the pH-stat system where the luminal pH and CO_2 (and therefore HCO_3^- concentration and its transepithelial gradient) are essentially clamped to a constant value.

Membrane-associated calcium sensing receptors are known from most organs in higher vertebrates [29], including the intestine [30], and are involved in bicarbonate secretion in the rat pancreatic duct [31]. With this in mind, and the calcium-sensitive bicarbonate secretion observed in the present study resulting in apparent control of luminal calcium concentrations, the involvement of a calcium sensing receptor in intestinal bicarbonate secretion is an appealing hypothesis. Calcium sensing receptors described to date respond to calcium concentrations in the millimolar range [29], which means that the calcium concentrations in the intestinal lumen of marine teleosts [10] correspond with the activation range of known calcium sensing receptors. Although these types of receptors exhibit differential responses to calcium and magnesium [32], the very high magnesium concentrations in the intestinal lumen of marine teleosts (as high as 170 mM; [9]) would present a challenging environment for calcium sensing.

An alternative mechanism of calcium stimulation of bicarbonate secretion in the marine teleost intestine could be via altered intracellular calcium concentrations as a result of alterations of luminal calcium. In the pancreatic duct epithelium intracellular calcium regulates bicarbonate secretion [33], and it is conceivable that elevated intracellular calcium resulting from elevated luminal calcium concentrations in the intestine of marine fish acts in a similar manner.

A dramatic stimulatory effect of elevated calcium on bicarbonate excretion was also observed over a 3-day period during in vivo perfusion of the intestine. The stimulation maintained over this longer time period suggests that the imbibed (intestinal) calcium is not only an important modulator of intestinal bicarbonate secretion, but that its precipitation may be a primary function of intestinal bicarbonate secretion in marine teleosts. The subsequent precipitation of 83% of the perfused calcium from the high calcium perfusate (Wilson, R.W. and Grosell, M., unpublished data) slightly more than accounts for the 'additional' 15 mM calcium in this saline. Thus, the rate of bicarbonate secretion seems to be well matched to rate of calcium entry into the intestine. The functional significance of matching bicarbonate secretion to calcium precipitation appears to lie in osmoregulation. Over the 72 h of the perfusion experiments, enhanced precipitation in the high calcium treatment did not significantly increase water absorption by the intestine. However, this would not be expected over such a long time scale if the enhanced precipitation of $CaCO_3$ simply results in the same transepithelial osmotic gradient for driving water absorption but at new, lower, steady state osmolalities within the plasma and intestinal fluids. This was indeed observed and for hypo-osmoregulating marine teleosts, the advantage of maintaining a lower plasma osmolality by such a mechanism is clear.

It is perhaps surprising that the osmolality of the intestinal fluid has such an influence on the extracellular fluid of the whole animal, given that the gills of fish in all three treatments were ventilating exactly the same seawater medium that was extremely hyper-osmotic to the plasma ($\sim$ 850 compared to $\sim$ 310 mOsm kg^{-1}) and at much higher flow rates (>1000-fold; [8]) than the intestine is normally perfused with. This presumably indicates a very large difference in the osmotic permeability of the gill and intestinal epithelia in marine fishes.

The 100-fold higher buffer capacity of the saline containing 5 mM Hepes did not affect the ability of the intestine to produce an alkaline and bicarbonate-rich rectal fluid. However, the proportion of these bicarbonate equivalents excreted as precipitated carbonate was half that of the control group, even though the total J_{HCO3} was essentially the same. This might suggest that buffering to pH 7.4 did act to inhibit the precipitation of calcium but only in the more anterior parts of the intestine, and given sufficient time as the perfusate proceeds along the intestine the buffering was overcome with the intestine ultimately acting to control pH and $[HCO_3]$ to set values in the excreted rectal fluid. Buffering the perfusate was therefore partially successful, in that the precipitation of calcium was reduced, and the subsequent effect on rectal fluid and plasma osmolality was in the direction predicted (higher), and significantly higher than the high calcium group ($P<0.006$), though not significantly different from the controls.

4.2. Conclusions

The stimulation of intestinal bicarbonate secretion by calcium both acutely and chronically, in vitro and in vivo, strongly indicates a direct functional link between bicarbonate secretion and the precipitation of divalent marine cations. Furthermore, for the first time it has been demonstrated that the precipitation of calcium caused by bicarbonate secretion within the intestine can significantly impact whole animal osmoregulatory status. Thus, the model presented here for a role of bicarbonate secretion in osmoregulation seems well founded. This model of net bicarbonate secretion promoting net water absorption in the opposite direction represents a previously unrecognised mechanism for facilitating epithelial water transport. The role of carbonate precipitation in water absorption is supplementary to the more traditionally accepted processes of net ion uptake followed by osmotically obliged water. The apparent ubiquity of intestinal bicarbonate secretion and carbonate precipitation in marine teleosts that hypo-osmoregulate, and its absence in osmo-conforming marine elasmobranchs and agnathans [6], does suggest that the supporting role it plays in osmoregulation is evolutionarily significant.

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