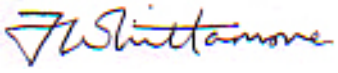
# The regulation of intestinal bicarbonate secretion by marine teleost fish

**Volume 2 (of 2)**

Submitted by Jonathan Mark Whittamore, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences in December 2008.



Signature  Date **10th December 2008**

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# Chapter Five

## The role of intestinal $HCO_3^-$ production, secretion and precipitation in fluid absorption and $Ca^{2+}$ homeostasis by a marine teleost

## 1. Summary

Drinking is a fundamental part of the osmoregulatory strategy for teleosts occupying a hyper-osmotic environment and the ability to effectively absorb fluid across the intestine is necessary to prevent dehydration and maintain hydromineral balance. Fluid absorption has previously been characterised as almost exclusively dependent on NaCl cotransport. However, not only can intestinal $HCO_3^-$ secretion drive fluid absorption directly (*via* $Cl^-/HCO_3^-$ exchange) but also indirectly by precipitating $Ca^{2+}$, and to a lesser extent $Mg^{2+}$, to their respective carbonates thereby removing the osmotic influence of these ions and enhancing the osmotic gradient for fluid absorption, thus representing a novel mechanism of water transport. The present study set out to further test this hypothesis and attempt to elucidate the role of carbonate precipitation in fluid absorption and $Ca^{2+}$ homeostasis by perfusing the European flounder intestine *in vivo* with salines containing various concentrations of $Ca^{2+}$, 10 mM (Control), 40 mM and 90 mM, to exaggerate $HCO_3^-$ secretion and precipitation. The rate of $CaCO_3$ precipitation increased more than 4-fold with only a modest increase in fluid absorption at 40 mM compared with the control treatment. However, at 90 mM $Ca^{2+}$, not only was the rate of precipitation even higher, but fluid absorption increased significantly (by more than 50 %), representing a dramatic increase in fluid transport. Despite the vastly higher rates of precipitation in the 40 mM and 90 mM treatments this did not lead to concomitant reductions in rectal fluid osmolality which were expected in order to drive this increased water absorption. However, since only the bulk fluid osmolality was measured it was impossible to detect the localised osmotic gradients that would be created by $CaCO_3$ precipitation within the mucus layer. In spite of this, the increase in fluid transport was independent of the net $Na^+$ flux thus negating a role for NaCl cotransport, but was instead dependent on net $Cl^-$ absorption. Analysis of the ion flux data revealed a missing cation, identified as $H^+$, based on strong correlations with the independent measurements of intestinal $HCO_3^-$ secretion and excretion of acidic equivalents to the external seawater. Not only did this analysis corroborate $H^+$ as the missing cation, but suggested that $HCO_3^-$ secretion was produced from the endogenous hydration of $CO_2$, even under hyper-stimulated conditions, and predicted that the fluid absorbed in association with $HCO_3^-$ secretion and precipitation would be hypo-osmotic to the perfusion saline thus accounting for the significant 18 mOsm $kg^{-1}$ reduction in

osmolality of the blood plasma. Based on this analysis, a model has been proposed explaining how $CO_2$ moves between various compartments of the intestine, linking $HCO_3^-$ production, secretion and $CaCO_3$ formation with fluid absorption. The present work therefore provides additional and convincing evidence for the participation of $HCO_3^-$ production, secretion and $CaCO_3$ precipitation in a novel mechanism of water transport by the marine teleost intestine, along with discussion of the role for carbonate precipitation in systemic $Ca^{2+}$ homeostasis.

## 2. Introduction

The ability of the intestine to absorb fluid is essential for all vertebrates, vital to replenishing losses of water and electrolytes during routine physiological processes such as digestion and also coping with extreme, dehydrating environments such as seawater. For marine teleosts, drinking is a key part of their strategy for replacing lost water and the intestine must therefore be capable of effectively absorbing fluid from imbibed seawater. Since the influential work of Homer Smith (1930) fluid transport by the intestine of marine fish has been associated with $Na^+$ and $Cl^-$, and some of the earliest *in vitro* and *in vivo* experiments on this tissue have shown that fluid can move in the absence of, or even against an osmotic gradient, linked to the energy-dependent absorption of NaCl (House and Green, 1965; Skadhauge, 1969).

### 2.1 A historical perspective on fluid transport

From previous studies of the isolated mammalian intestine, Curran and Solomon (1957) had first proposed that water absorption was passive, following the active transport of solutes ($Na^+$ and $Cl^-$) from the lumen. In addition, absorption was also found to take place against a transepithelial osmotic gradient (Parsons and Wingate, 1961a), and the absorbed fluid was characteristically iso-osmotic to the bathing medium (Curran, 1960). These observations all led up to the publication of a simple three compartment, double membrane model by Curran and MacIntosh (1962). This system proposed a hyper-osmotic, intra-epithelial compartment arising from active solute transport across the outer membrane, which would passively draw water into this compartment, and following a consequent rise

in hydrostatic pressure, force the absorbed fluid out across a second inner membrane. The identity of this intra-epithelial compartment was suggested by Whitlock and Wheeler (1964) as being analogous to the lateral inter-cellular space (LIS). These ideas were subsequently expanded and refined by Diamond and Bossert (1967) to produce their "standing gradient hypothesis" of solute-linked water transport. This described active solute transport into the LIS which establishes a localised region of hyper-osmolarity next to the tight junction drawing in water from adjacent cells and creating a standing osmotic gradient along the LIS, with the increased hydrostatic pressure forcing the fluid out across the basement membrane.

While many of the assumptions and predictions made by this model which include the clustering of solute pumps ($Na^+$-$K^+$-ATPase) at the tight junction end of the LIS, the relatively low hydraulic permeability of the lateral membranes and the possibility of 'leaky' tight junctions, are now considered unnecessary or incorrect (reviewed by Schultz, 1998; Spring 1998; 1999), the basic features of this model still form the dominant viewpoint of how water is absorbed across epithelia like the intestine. Even though developments in molecular and cell biology have significantly enhanced our understanding of water transport, the most prominent being the discovery of transmembrane water channels, known as aquaporins (Preston *et al*., 1992; Agre *et al*., 2002), there still remain questions and controversies underlying the specific mechanisms and pathways of water transport (Spring, 1999; Schultz, 2001; Reuss and Hirst, 2002; Spring, 2002; Chapter 1, Section 3.3). Despite this the vast majority of physiologists accept that the driving force for fluid transport are localised osmotic gradients across the cell membrane, where the movement of water is considered: a) passive, b) secondary to net solute transport in the same direction

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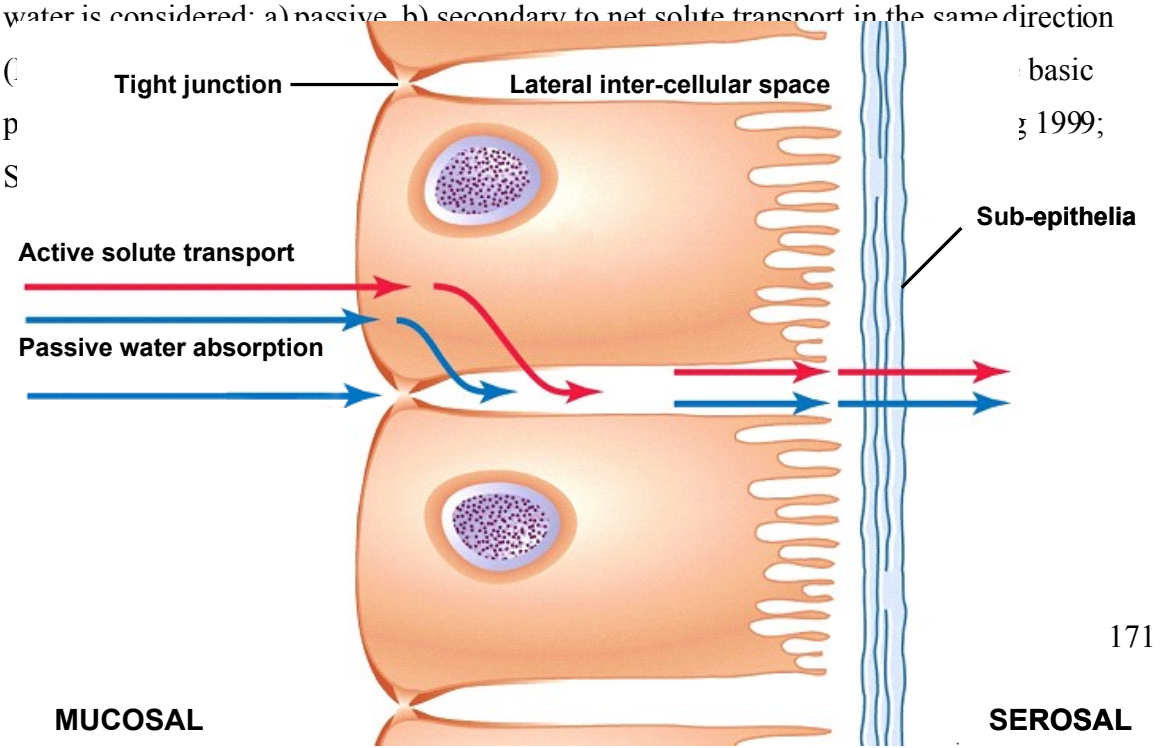


Figure 5.1: A simple illustration of solute-coupled fluid absorption across an epithelia, where solutes (red arrows) from the lumen will enter the cell across the apical membrane (e.g. *via* NaCl co-transport) and moveacross the basolateral membrane into the lateral inter-cellular space (LIS) creating a region of localised hyper-osmolarity. Water (blue arrows) follows passively along the osmotic gradient through either transmembrane pathways (or potentially across the tight junction) and iso-osmotic fluid exits at the base of the LIS (Modified from Randall *et al.*, 1997).

**2.2 The mechanism of intestinal fluid transport in teleosts**

The ideas behind the solute-coupling model shown in Figure 5.1, and the involvement of $Na^+$-coupled transport (i.e. apical NaCl cotransport), were largely the product of original experimental observations on mammalian epithelia such as the gall bladder (Diamond, 1964; Whitlock and Wheeler, 1964), small intestine (Curran and Solomon, 1957; Curran, 1960; Parsons and Wingate, 1961b) and renal proximal tubule (Green and Giebisch, 1984). By comparison, the mechanism of fluid transport is not considered to be exceptionally different in the marine teleost intestine that receives large amounts of $Na^+$ and $Cl^-$ from imbibed seawater (House and Green, 1965; Skadhauge, 1969; 1974), and in terms of hypo-osmoregulation has been characterised as almost exclusively absorbing NaCl (Loretz, 1995; Karnaky, 1998), accommodating the characteristics of the solute-linked water transport model discussed above.

However, drinking seawater presents the gastrointestinal tract with the unique challenge of not only dealing with high concentrations of $Na^+$ and $Cl^-$, but also coping with large amounts of divalent ions ($Ca^{2+}$, $Mg^{2+}$ and $SO_4^{2-}$) that are also present. The bicarbonate-rich, alkaline nature of the intestinal fluids (pH 8-9) created by $HCO_3^-$ secretion are quite different from mammals (~pH 7.4) and are a prominent feature of teleosts occupying a hyper-osmotic environment (Wilson, 1999; Grosell, 2006), and to some extent for all fish that drink seawater (Taylor and Grosell, 2006b). Despite observations of alkaline intestinal fluids and precipitated calcium carbonate ($CaCO_3$) from as far back as the work of Smith (1930), who incidentally wrote that they were of "no significance", it is only in the past two decades that there has been considerable insight made into the novel, functional aspects of $HCO_3^-$ secretion and $CaCO_3$ precipitation.

**2.3 The role of intestinal $HCO_3^-$ secretion and precipitation in fluid transport**

For almost all species examined so far the secretion of $HCO_3^-$ is mediated by apical $Cl^-/HCO_3^-$ exchange (Ando and Subramanyam, 1990; Grosell and Jensen, 1999; Grosell *et al.*, 2001; Wilson *et al.*, 2002; Grosell *et al.*, 2005; Kurita *et al.*, 2008), produced largely endogenously from the hydration of $CO_2$ within the epithelia (Wilson *et al.*, 1996; Wilson and Grosell, 2003; Grosell *et al.*, 2005; Grosell and Genz, 2006; Present study). In addition to the protons ($H^+$) yielded from the hydration of $CO_2$, the exchange of $HCO_3^-$ for $Cl^-$ will result in a net gain of osmolytes by the cell (since the osmotic pressure exerted by $CO_2$ is negligible), and therefore makes a direct contribution to solute-linked water absorption, supplementary to NaCl cotransport, as demonstrated for the European flounder (Grosell *et al.*, 2005).

Compared to $Na^+$ and $Cl^-$, the divalent ions ($Ca^{2+}$, $Mg^{2+}$ and $SO_4^{2-}$) are poorly absorbed along the intestine, and as a consequence of solute-linked water transport they become increasingly concentrated. However, the alkaline nature of the intestinal fluids support the production of carbonate precipitates which were found to be rich in $Ca^{2+}$ (and to a lesser extent $Mg^{2+}$). Having eliminated specific roles in relation to feeding and acid-base regulation, it was suggested their formation was involved in the process of osmoregulation by preventing the build-up of these ions which would otherwise accumulate with adverse effects on the osmotic gradient for fluid absorption (Humbert *et al.*, 1986; Walsh *et al.*, 1991; Wilson *et al.*, 1996).

A simple theoretical treatment, based on the concentrations of ions entering and leaving the gut, showed that by secreting $HCO_3^-$ and removing $Ca^{2+}$ as $CaCO_3$, the ion content of the intestinal fluid could be reduced by approximately 70 mM, thus offering a considerable osmotic advantage in terms of promoting fluid absorption (Wilson *et al.*, 2002). In addition to the direct role of $Cl^-/HCO_3^-$ exchange in fluid absorption by the teleost intestine (Grosell *et al.*, 2005), it was hypothesised that the production of carbonate precipitates would also have an indirect role in facilitating water absorption (Wilson *et al.*, 2002). What makes this idea unique in terms of the accepted understanding of fluid transport is that it does not rely on net solute absorption, followed by osmotically obliged water in the same direction, but rather the precipitation of $CaCO_3$ creates the osmotic gradient for water absorption by reducing the osmolality of the fluid within the intestine, therefore representing a novel mechanism of water transport in vertebrate epithelia (Wilson *et al.*, 2002).

Wilson *et al.* (2002) proceeded to put these observations to the test, presenting the first evidence linking $HCO_3^-$ secretion, carbonate precipitation and osmoregulation after perfusing the intestine of the European flounder *in vivo* with saline containing 20 mM $Ca^{2+}$, to deliberately stimulate $HCO_3^-$ secretion and precipitation, and collecting the expelled fluid and precipitates. Overall, $HCO_3^-$ production and secretion was increased by 57 % (compared to a control perfusion saline containing 5 mM $Ca^{2+}$). Almost all the additional 15 mM $Ca^{2+}$ could be accounted for as $CaCO_3$ and was associated with a significant reduction in the osmolality of the gut fluid as predicted, and interestingly, a reduction in blood plasma osmolality. However, after 72 hours of perfusion there was only a small 4 % increase in water absorption which was not significantly different from the control.

The proposal of a novel mechanism of water transport is an intriguing possibility but there still remains only indirect evidence in support of this hypothesis. Using the same perfusion technique as Wilson *et al.* (2002), the present study set out to exaggerate the process of precipitation even further by perfusing the intestine with higher concentrations of $Ca^{2+}$, 40 mM and 90 mM, along with 10 mM $Ca^{2+}$ as a control, in an attempt to try and resolve the effects of $CaCO_3$ production on fluid transport. These $Ca^{2+}$ concentrations would be equivalent to the amount being received by the intestine if the fish were drinking normal (10 mM $Ca^{2+}$), double (20 mM $Ca^{2+}$) and triple (30 mM $Ca^{2+}$) strength seawater and this was being matched by a proportional increase in drinking rate (i.e. $20 \times 2 = 40$ mM, and $30 \times 3 = 90$ mM).

### 2.4 The role of $CaCO_3$ precipitation in $Ca^{2+}$ homeostasis

The chemistry of $Ca^{2+}$ is unique among the elements, possessing a set of chemical and physical properties which enable the ion to interact with a multitude of organic and inorganic molecules, thus conferring a diverse set of functions within the body (Williams, 1970; Jaiswal, 2001). Like many vertebrates, the pool of $Ca^{2+}$ within teleosts is dominated by the skeleton where it is a key structural component of bone, as well as otoliths and teeth, with substantial deposits also found in the skin and scales (Fleming, 1973; Simkiss, 1973). The smallest fraction (approximately 1-3%) of total body $Ca^{2+}$ is found as an exchangeable pool within the intra- and extracellular fluids, actively participating in a broad range of crucial functions including muscle contraction, neurotransmission, enzyme and hormone secretion as well as initiating and co-ordinating numerous cellular processes (Fleming *et al*., 1973; Rubin, 1982). The ubiquitous nature of this ion therefore requires an effective homeostatic system since any disturbances are liable to compromise key functions (Brown *et al*., 1995).

Seawater contains approximately 10 mM $Ca^{2+}$ and with concentrations in the blood and extracellular fluids typically maintained at 2-3 mM, marine teleosts living in and drinking the surrounding water are constantly faced with an influx of $Ca^{2+}$ into the body. The normal pathway for $Ca^{2+}$ excretion in freshwater fish is *via* the kidneys (Karnaky, 1998; Marshall and Grosell, 2006), but marine teleosts, faced with the challenge of water conservation in dehydrating environment have a very low rate of urine production relative to drinking rates. The urinary bladder provides the opportunity to absorb additional fluid to reduce overall water losses the end result being an infrequent release of small volumes of concentrated urine (Chapter 1, Section 2.2.5).

The demonstration by Nearing *et al*. (2002) that a calcium-sensing receptor (CaR) modulates fluid re-absorption by the bladder to avoid the potential precipitation of divalent ions further illustrates the limited capacity of the renal system to operate as a pathway for $Ca^{2+}$ excretion. As the primary sites of contact with the surrounding seawater the gills and intestine must therefore be very effective at regulating the entry of $Ca^{2+}$ into the body. In terms of this homeostasis the precipitation of $CaCO_3$ helps to limit the availability of $Ca^{2+}$ for absorption where it has been shown to remove between 40-60 % of imbibed $Ca^{2+}$, which increases proportionally with up to 25 mM $Ca^{2+}$ entering the intestine (Shehadeh and

Gordon, 1969; Wilson *et al*., 2002; Wilson and Grosell, 2003). Although flounder have been shown to tolerate up to an additional 70 mM $Ca^{2+}$ in surrounding seawater (Wilson and Grosell, 2003), there was no accompanying description of the fate of intestinal $Ca^{2+}$ in these particular animals. With plans to perfuse the intestine with up to 90 mM $Ca^{2+}$ in an attempt to determine the role of $CaCO_3$ precipitation in water transport, it would also be interesting to further examine its additional role in systemic $Ca^{2+}$ homeostasis, and how the intestine, and indeed the animal as a whole copes with sustained exposure to high concentrations of intestinal $Ca^{2+}$.

## 3. Materials and Methods

### 3.1 Experimental animals

European flounder, *Platichthys flesus* (mean body mass 465 ±31 g, n = 23) were obtained from local fishermen in Flookburgh, Cumbria, U.K. and transported to the School of Biosciences, University of Exeter where they were held in marine aquarium facilities in 150 litre tanks of flowing, aerated, artificial seawater made with commercial marine salts (Tropic Marin), as part of a recirculating seawater system maintained at 33.8 ±0.2 ppt and 12.5 ±0.3 °C, under a 12 hour light: dark photoperiod. At least 7 days were allowed for the fish to acclimate after arriving in the aquarium. Food was typically withheld for at least 72 hours prior to experimentation, otherwise the fish were maintained on a diet of fresh ragworm (*Nereis virens*) fed once per week.

### 3.2 *In vivo* surgical procedures

To prepare for surgery each fish was anaesthetised in seawater containing 150 mg $l^{-1}$ of tricaine methansulfonate (MS-222, Pharmaq Ltd.), buffered with 300 mg $l^{-1}$ $NaHCO_3$, before being placed on a custom-made wet table. To maintain anaesthesia during surgery the gills were constantly irrigated with aerated seawater containing 95 mg $l^{-1}$ buffered tricaine methansulfonate pumped into the mouth (Plate 5.1A). Three separate procedures were subsequently performed in the following order:

#### 3.2.1 Cannulation of the caudal blood vessel

Implanting a catheter into the caudal blood vessel enabled blood sampling at regular intervals during the course of a perfusion in order to assess ion and acid-base parameters. The catheter was made from a 25 cm length of polyethylene (PE) tubing (ID = 0.58 mm, OD = 0.96 mm) filled with Cortland saline (Wolf, 1963), adjusted to pH 7.8 with NaOH and containing 50 i.u. $ml^{-1}$ sodium heparin (Monoparin, Wockhardt UK Ltd.). After gently scraping the scales from a small area close to the tail, a 2 cm incision was made through the skin and muscle parallel to the lateral line to reveal the caudal vessels running side by side beneath the vertebral spines (Plate 5.1B). The catheter was then inserted randomly into either the artery or vein. To help secure the catheter, a grommet was made from a short, 2 cm length of PE tubing (ID = 1.19 mm, OD = 1.70 mm) which was slid down the outside of the catheter and fixed in place with a drop of cyanoacrylate adhesive. After treating the area with a prophylactic antibiotic the incision was then closed around the grommet using a basic running stitch (Plate 5.1C). Following successful cannulation of the caudal blood vessel, a small blood sample was taken from each fish and centrifuged (MSE Microcentaur) at 11,600 × g for 3 min, and plasma osmolality measured by vapour pressure osmometer (Wescor Vapro 5520). The osmotic pressure of the perfusion saline was then adjusted (if necessary) with deionised water to match that of the blood plasma.

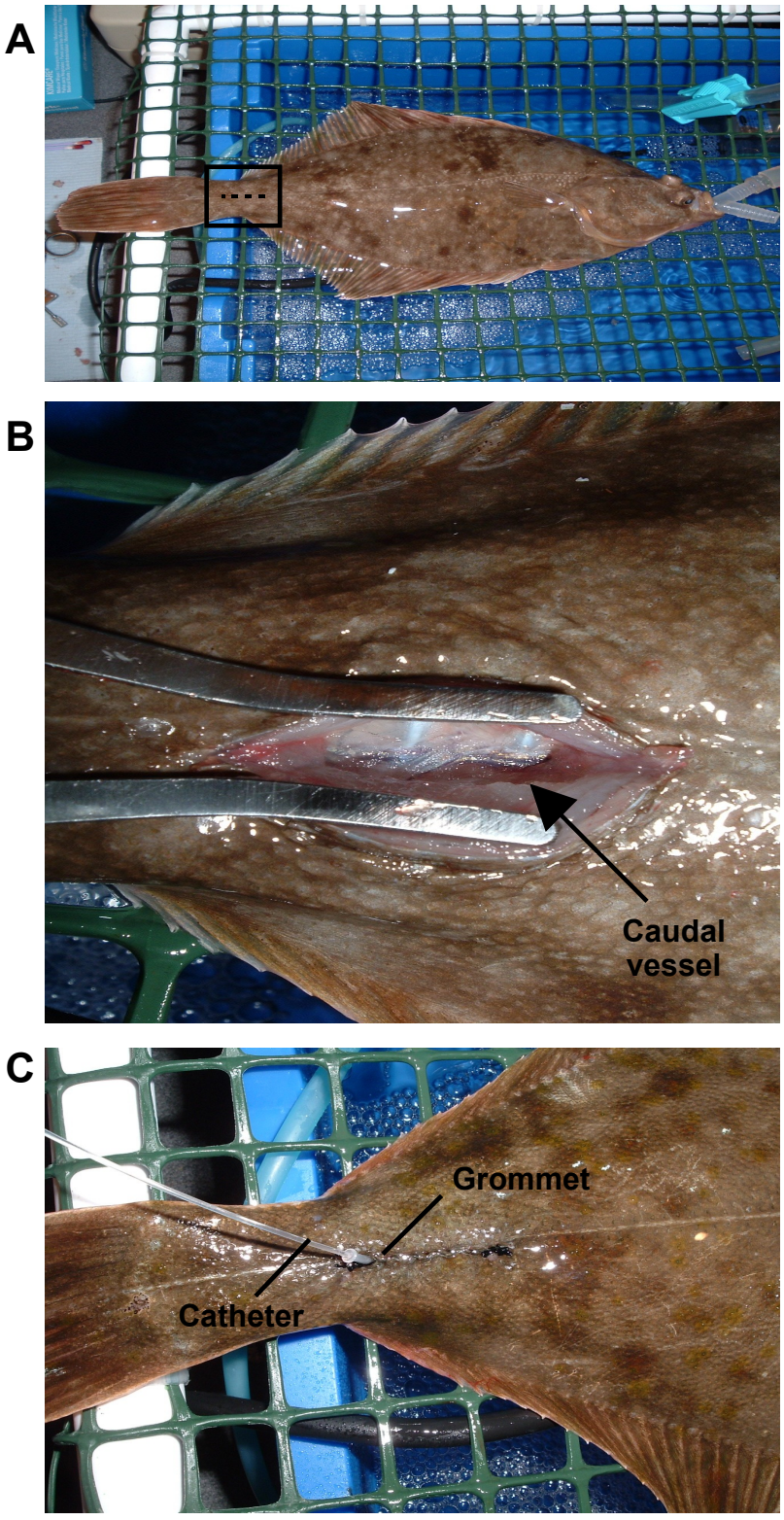



Plate 5.1: A) A flounder under anaesthesia on the wet table the gills being irrigated *via* the mouth. The black square indicates the location of the blood catheter, and the dashed line

the approximate site of the incision. B) After making the incision next to the lateral line the wound is held open to reveal the blood vessels (arrow) running alongside the vertebrae. C) A close-up of the implanted blood catheter being held securely in position by the grommet after the wound had been closed.

### 3.2.2 Fitting the intestinal perfusion and stomach drain catheters

In addition to the intestinal catheter, a stomach drain catheter was also fitted since the fish was going to continue drinking during these experiments and it was important that this imbibed fluid was able to drain away. Both the stomach and intestinal catheters were cut from PE tubing (ID = 1.19, OD = 1.70) to lengths of 8-10 cm and heat-flared at one end. A small 2 cm long incision was made in the upper area of the abdominal cavity, perpendicular to the lateral line and just behind the pectoral fin. The junction between the stomach and intestine was located and exposed through the incision (Plate 5.2A). A second, smaller incision was made in the stomach wall behind the pyloric sphincter, through which the flared end of the stomach drain catheter was gently pushed into the stomach and directed towards the oesophagus. The intestinal catheter was inserted *via* the same incision, but in the opposite direction, through the pyloric sphincter and into the anterior intestine. Taking care to avoid damaging or occluding any blood vessels, both catheters were held firmly in place by double silk ligatures tied around the stomach wall and the pyloric sphincter for the stomach drain and intestinal perfusion catheter, respectively (Plate 5.2B). The stomach and intestine were carefully pushed back into the abdominal cavity and the incision closed around the catheters with a basic running stitch after treating the area with prophylactic antibiotic (Plate 5.2C). The intestine was then flushed with 50 ml of relevant perfusion saline through the intestinal catheter, while gently massaging the abdomen, to thoroughly rinse out the existing gut fluid and any precipitates before attaching the rectal catheter.

### 3.2.3 Fitting the rectal catheter

The final procedure was to fit a catheter bag to collect all the fluid and precipitates expelled from the rectum. This was made using a condom, where the open end was tied tightly around a 1 cm length of ridged, cone-shaped plastic tubing (ranging from ID = 4

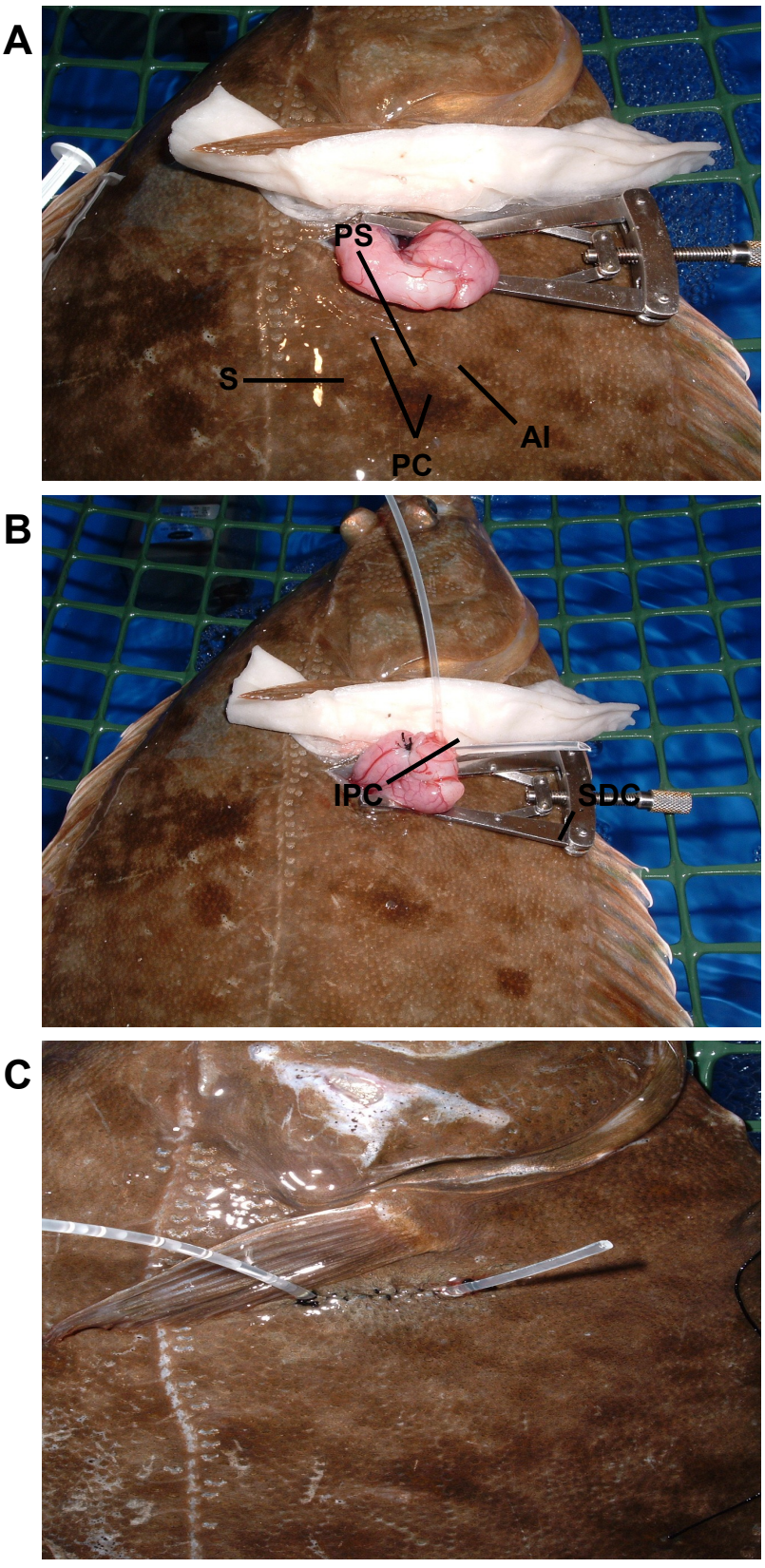

A
PS
S
PC
AI
B
IPC
SDC
C

Plate 5.2: A) The stomach (S) and anterior intestine (AI), separated by the pyloric sphincter (PS), exposed through an incision made in the abdominal cavity as observed looking toward the head end of the fish. The site of the second, smaller incision into the stomach wall is marked by an arrow. A white medical tissue acts as a barrier protecting the wound from coming into contact with seawater exiting the operculum. Note the vestigial pyloric caecae (PC) of the anterior intestine. B) The stomach drain (SDC) and intestinal perfusion catheters (IPC) inserted into the stomach and intestine, respectively, *via* an incision in the stomach wall. C) After carefully replacing the catheterised portions of the gastrointestinal tract back into the abdominal cavity the incision was closed.

mm, OD = 7 mm; ID = 6 mm, OD = 8 mm or ID = 7 mm, OD = 10 mm, depending on the size of the fish). A small nick was made in the closed end of the condom and a 3-way stopcock (Vygon) tied into place allowing excess rectal fluid to be drained should the catheter become over-full during the course of a perfusion (Plate 5.3A). The catheter was fitted by inserting the ridged plastic tubing into the anus which was held in place by a purse string ligature sewn into the skin around the anal opening (Plate 5.3B). To help keep the catheter in place and prevent it from working loose it was attached to the anal fin by a ligature around one arm of the 3-way stopcock (Plate 5.3C). To minimise the risk of infection the incisions made for implanting the blood and intestinal catheters were treated with a topical prophylactic antibiotic solution, containing oxytetracycline (Sigma) dissolved in Cortland saline, before closure of the wound. All sutures and ligatures used wax-coated, braided silk suture thread (Pearsalls, US 2/0).

**3.3 Saline composition and experimental design**

The perfusion salines used in the present study were modified from Wilson *et al*. (2002) and designed to test the influence of $Ca^{2+}$ on $HCO_3^-$ secretion and precipitation rates *in vivo*, as well as the simultaneous effects of these processes on intestinal fluid absorption and osmoregulation. Fish were allocated to one of three treatment groups (shown in Table 5.1), which consisted of a control saline containing 10 mM $Ca^{2+}$, similar to the concentration entering the anterior intestine while drinking normal seawater. To enhance $CaCO_3$ precipitation, the second group were perfused with 40 mM $Ca^{2+}$ and the third with

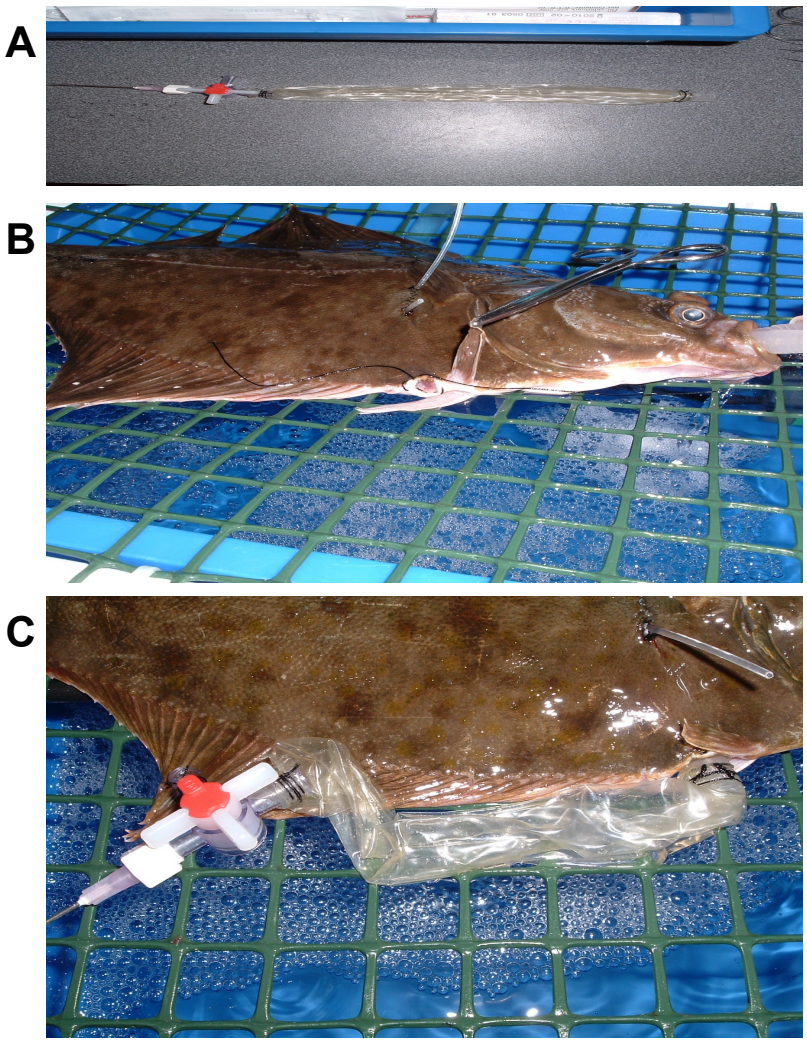



Plate 5.3: A) The rectal catheter bag showing the ridged section of plastic tubing that forms the opening which is inserted into the anus and, at the opposing end a 3-way stopcock allowing the catheter to be drained, if necessary. B) The purse string ligature sewn around the anal opening ready to secure the catheter in place. C) The fitted rectal catheter.

90 mM $Ca^{2+}$, thus approximating the amount of $Ca^{2+}$ received by the intestine if the fish were occupying double and triple strength seawater, respectively, where drinking rates would have also increased in direct proportion. In order to preserve the $Cl^-$ concentration between these salines when manipulating $Ca^{2+}$, $MgCl_2$ was exchanged for $CaCl_2$. To prevent the spontaneous precipitation of $CaSO_4$ in the presence of such high $Ca^{2+}$ concentrations, $SO_4^{2-}$ was reduced to 10 mM. Also, each saline was deliberately $HCO_3^-$ free

and unbuffered, therefore the appearance of any $HCO_3^-$ and precipitates could have only originated from the activity of the perfused intestine.

Table 5.1: The inorganic salts used in the composition of the *in vivo* perfusion salines employed by the present study. The concentration of each component salt is given in mmol $l^{-1}$. Theoretical osmolarity (mOsm $l^{-1}$) was calculated based on the osmotic coefficient of each salt (Robinson and Stokes, 1965).

| Salt | Perfusion treatment | | |
|---|---|---|---|
| | Control | 40 mM $Ca^{2+}$ | 90 mM $Ca^{2+}$ |
| NaCl | 50.0 | 50.0 | 50.0 |
| KCl | 5.0 | 5.0 | 5.0 |
| $MgCl_2.6H_2O$ | 80.0 | 50.0 | - |
| $CaCl_2.6H_2O$ | 10.0 | 40.0 | 90.0 |
| $MgSO_4.7H_2O$ | 10.0 | 10.0 | 10.0 |
| Osmolarity | 353 | 350 | 346 |

### 3.4 Intestinal perfusion

Once surgery was complete the fish was placed in an individual flux chamber (7 litre capacity) with a continuous supply of aerated seawater in order to recover from the anaesthesia. During this recovery period the intestinal catheter was connected to a peristaltic pump (Minipuls 3, Gilson) and perfusion commenced with one of the three salines listed in Table 5.1 containing different concentrations of $Ca^{2+}$. The intestine of each fish was continuously perfused for 72 hours at a mean perfusion rate of 5.04 ±0.24 ml $kg^{-1}$ $h^{-1}$ (n = 23), approximately two and a half times the normal drinking rate of flounder, cited as 2.02 ±0.36 ml $kg^{-1}$ $h^{-1}$ (Wilson *et al.*, 2002), thus ensuring an adequate supply of fluid to the intestine. The duration of each perfusion was 72 hours, this permitted sufficient time for precipitates to accumulate since this process was likely to be relatively slow at the temperatures employed here (11-12 °C). The volumes perfused were determined by the difference in weight to the nearest 0.1 mg (Mettler AE163).

### 3.5 Sampling and analytical techniques

Blood samples (~800 µl) were taken at 24, 48 and 72 hours using a gas-tight 1 ml Hamilton syringe and processed immediately. Plasma was isolated from approximately 500 µl of

blood by centrifuging (11,600 × g for 3 min) and was subsequently stored on ice. The remaining 300 µl was used to measure whole blood pH with Cameron E301 glass electrode with an E351 reference electrode connected to an Alpha 600 ion meter. This system was enclosed in a water jacket and maintained at the experimental water temperature (12.5 ±0.3 °C). Once pH had been measured this blood was returned to the animal along with 500 µl of Cortland saline (adjusted to pH 7.8 with NaOH), to replace the volume taken and filling the blood catheter with heparinised Cortland saline to reduce the risk of clot formation. After 72 hours the experiment was terminated by stopping the intestinal perfusion pump and administering an overdose of anaesthetic (250 mg $l^{-1}$ buffered MS-222) into the flux chamber. The perfusion catheter was then detached from the peristaltic pump and a ligature tied around the top of the rectal catheter before removing the fish from the chamber. The abdominal cavity was opened and another ligature tied around the base of the rectum to prevent the accidental loss of any contents. The intestine was carefully dissected out and the contents decanted into 50 ml centrifuge tubes. Similarly, the rectal catheter was removed and its contents collected. Any residual precipitates were obtained by rinsing the intestine.

The contents of the intestine and rectal catheter were centrifuged at 5,000 × g for 4 min at 4 °C (Mistral 3000, MSE Scientific Instruments) separating the precipitates from the fluid, the latter being decanted into pre-weighed tubes and subsequently weighed to determine the volume of fluid retrieved. Measurements of osmolality (Wescor Vapro 5520), $Cl^-$ (Corning chloride analyser 925) and total $CO_2$ (Mettler Toledo 965D carbon dioxide analyser) were made on all samples. The cations $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ were also measured following appropriate dilution and addition of 1 % $LaCl_3$ (as 10 % w/v solution) for the divalent ions using a PYE Unicam SP9 Atomic absorption spectrophotometer. The pH of the intestinal and rectal fluids were measured using an Accumet combined microelectrode connected to a Hanna HI8314 membrane pH meter.

The precipitates from the intestine and rectal catheter of each fish were pooled together, and rinsed three times in deionised water, re-centrifuging each time, before they were finally sonicated (Vibra-Cell, Sonics and Materials Inc.) in 20 ml of ultrapure water (Maxima Ultrapure Water, ELGA), measured by glass pipette. The content of bicarbonate equivalents ($HCO_3^- + 2CO_3^{2-}$) in the precipitates was determined by the double titration method (described previously by Wilson *et al.* (2002) and Wilson and Grosell (2003)).

Briefly, each sample was gassed with 100 % $N_2$ and the initial pH recorded before the addition of 1 N HCl. Continuous gassing ensured that all the $HCO_3^-$ and $CO_3^{2-}$ were liberated from the sample as $CO_2$ as it was acidified to pH 3.89. Once the pH had stabilised at 3.89 the volume of HCl added was recorded before the addition of 0.02 N NaOH returning the sample to its original starting pH. The difference in the number of moles of HCl and NaOH used to acidify and then return to the initial pH was considered equivalent to the number of moles of $HCO_3^-$ equivalents ($HCO_3^- + 2CO_3^{2-}$) present in the sample. An auto-titrator (TIM 845 titration manager with an SAC80 automated sample changer, Radiometer) was used for all double titrations, employing the same combination pH electrode (Red Rod Combined pH electrode, pHC2401-8, Radiometer), in conjunction with the same burettes (ABU52, Radiometer) delivering controlled amounts of acid and base. All pH readings (to within ±0.002 pH units) and volumes of acid and base added (to the nearest µl) were logged on a personal computer using Titramaster 85 software (version 3.1). To determine the amounts of $Ca^{2+}$ and $Mg^{2+}$ contained within the precipitates, the titrated sample was re-acidified to below pH 4 by adding a known volume of 0.02 N HCl (approximately equivalent to the volume of 0.02 N NaOH added during the double titration) and a 1 ml aliquot taken for subsequent dilution and cation analysis using AAS.

**3.6 Calculations**

For the determination of bicarbonate equivalents ($HCO_3^-$ and $2CO_3^{2-}$) in the intestinal and rectal fluids, the $HCO_3^-$ concentration was initially calculated from a similar re-arrangement of the Henderson-Hasselbach equation presented in Chapter 4 (Section 3.4) but substituting $pK_{app}$ for a second dissociation constant, $pK_{II}$ (9.46) which describes the $HCO_3^-/CO_3^{2-}$ reaction (1). This value is similar to the published $pK_{II}$ value of 9.52 for ⅓ seawater at the same temperature (Walton Smith, 1974), but was obtained empirically by Wilson *et al.* (2002) using the rectal fluids of the flounder and directly comparing the results of the double titration method with calculations based on measurements of $TCO_2$ and pH using the rectal fluids of the flounder.

$$[HCO_3^-] = [TCO_2] / (1 + 10^{(pH - 9.46)}) \qquad (1)$$

As mentioned previously, the total $CO_2$ content (mM) of a sample typically comprises:

$$[TCO_2] = [\text{molecular } CO_2] + [HCO_3^-] + [CO_3^{2-}] \quad (2)$$

The soluble and gaseous $CO_2$ fractions in the sample are represented by [molecular $CO_2$]. At the pH of the intestinal and rectal fluids the contribution of molecular $CO_2$ to the measurement of $TCO_2$ in these samples is very small and typically less than $10^{-9}$ M (Grosell *et al*., 2005), therefore in the present study the carbonate ($CO_3^{2-}$) fraction was subsequently calculated following rearrangement of equation 2 (3a), along with total bicarbonate equivalents (3b):

$$[CO_3^{2-}] = [TCO_2] - [HCO_3^-] \quad (3a)$$

$$\text{Total } HCO_3^- \text{ equivalents} = [HCO_3^-] + 2[CO_3^{2-}] \quad (3b)$$

The overall net secretion rate of total bicarbonate equivalents ($HCO_3^- + 2CO_3^{2-}$) by the intestine was calculated as:

$$J_{HCO3-} = [HCO_3^- \text{ Eq.}]_{IF} + [HCO_3^- \text{ Eq.}]_{RF} + [HCO_3^- \text{ Eq.}]_{PPT} / (M \times t) \quad (4)$$

Where, $[HCO_3^- \text{ Eq.}]_{IF}$, $[HCO_3^- \text{ Eq.}]_{RF}$ and $[HCO_3^- \text{ Eq}]_{PPT}$ represent the total concentration of bicarbonate equivalents measured in the intestinal fluid, rectal fluid and precipitates, respectively (mEq), M is the mass of the fish (kg), t is the perfusion time period (h) and $J_{HCO3-}$ the net secretion rate of bicarbonate equivalents ($\mu$Eq $kg^{-1}$ $h^{-1}$).

### 3.7 Data presentation and statistical analysis

The data are presented as mean ±SE. All data expressed as a proportion were arcsine transformed prior to analysis and significant differences between treatments tested for by one-way ANOVA using a General Linear Modelling (GLM) procedure. Post-hoc, pair-wise comparisons were made using Bonferroni simultaneous tests. For data failing to meet the assumptions of approximate normality and equality of variance, the non-parametric Kruskal-Wallis test was performed with post-hoc comparisons made using Dunns

procedure. The results of all tests were accepted as significant at $P<0.05$. Statistical analysis was carried out using Minitab v13.1 and graphs drawn using SigmaPlot v9.0.

## 4. Results

### 4.1 Bicarbonate production and excretion

Increasing the concentration of $Ca^{2+}$ perfusing the intestine produced a sustained elevation in the total amount of $HCO_3^-$ equivalents excreted and dramatically altered the rate of precipitate formation. At 40 mM there was a significant 31 % increase in total $HCO_3^-$ secretion accompanied by a more than 4-fold rise in the amount excreted as precipitates. When $Ca^{2+}$ was increased to 90 mM almost all (98 %) of the $HCO_3^-$ produced was incorporated into precipitates, at approximately twice the rate of the previous 40 mM $Ca^{2+}$ treatment and almost 8 times higher than the control (Figure 5.2).

The acid-base characteristics of the voided rectal fluid from flounder that underwent perfusion with high $Ca^{2+}$ were quite different compared with the control treatment. Rectal fluid pH under control conditions was alkaline and rich in $HCO_3^-$ equivalents. Increasing the concentration of $Ca^{2+}$ in the perfusion saline up to 40 mM reduced pH by 0.35 units and measured $TCO_2$ by almost 50 %. At 90 mM there was little alkalinisation of the rectal fluid with a pH of 7.41 and only small amounts of dissolved $HCO_3^-$ equivalents present (Table 5.2).

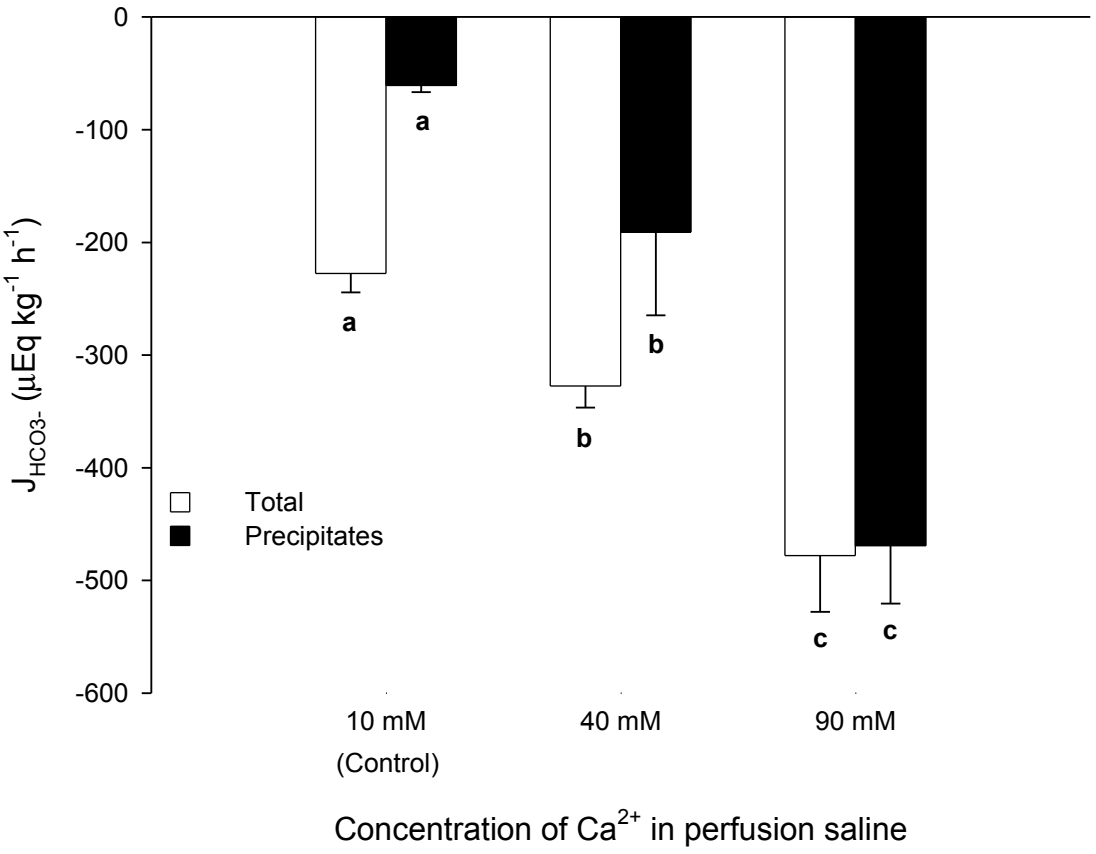



Figure 5.2: The mean (±SE) net production and excretion of bicarbonate ($HCO_3^-$ + $CO_3^{2-}$) equivalents (µEq $kg^{-1}$ $h^{-1}$) by the intestine of the flounder perfused with salines containing varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM over a total of 3 days. The open bars represent the total amount of bicarbonate equivalents (intestinal fluid + rectal fluid + precipitates) produced and shaded bars show the amount incorporated into precipitates only. Means labelled with different letters indicates a significant difference ($P \leq 0.05$), n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively.

Table 5.2: The mean (±SE) values for measurements of pH, $TCO_2$ (mM) and calculated $HCO_3^-$ equivalents (mEq) measured in rectal fluid samples from the flounder following perfusion of the intestine with salines containing varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM for 3 days. Means labelled with different letters indicate

a significant difference ($P < 0.05$), n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively.

| | Perfusion treatment | | |
|---|---|---|---|
| | **10 mM $Ca^{2+}$ (Control)** | **40 mM $Ca^{2+}$** | **90 mM $Ca^{2+}$** |
| pH | 8.57 (±0.03)[a] | 8.22 (±0.11)[b] | 7.41 (±0.11)[c] |
| $TCO_2$ (mM) | 61.2 (±4.7)[a] | 34.5 (±6.2)[b] | 6.6 (±1.3)[c] |
| $[HCO_3^- + 2CO_3^{2-}]$ (mEq $l^{-1}$) | 68.4 (±5.6)[a] | 37.3 (±7.3)[b] | 6.6 (±1.3)[c] |

### 4.2 Rectal fluid osmolality

Despite the vast differences in $HCO_3^-$ secretion and precipitation rates between all three treatments the resultant osmotic pressure of rectal fluids collected from fish undergoing the 90 mM treatment was rather variable and fell by an average of only 4 mOsm $kg^{-1}$. The reduction in osmolality of the rectal fluid (relative to perfusate) was more than twice as much (8-10 mOsm $kg^{-1}$) in the control and 40 mM treatments. Despite this there were no significant differences between rectal fluid and perfusate osmolality within each treatment (assessed by paired t-tests), or rectal fluid osmolality between treatments (Figure 5.3).

### 4.3 Fluid transport

Over 3 days the proportion of fluid absorbed by the intestine had increased by 11 % between the control and 40 mM treatments (from 47.2 ±3.5 % to 58.5 ±2.1 %) but was not found to be significantly different. When the intestine was perfused with 90 mM $Ca^{2+}$ fluid absorption increased even further reaching an average of 73 %, and although this was not significantly different from the 40 mM treatment it represented a very significant ($P < 0.001$) increase in relation to the control treatment (Figure 5.4). The values for fluid

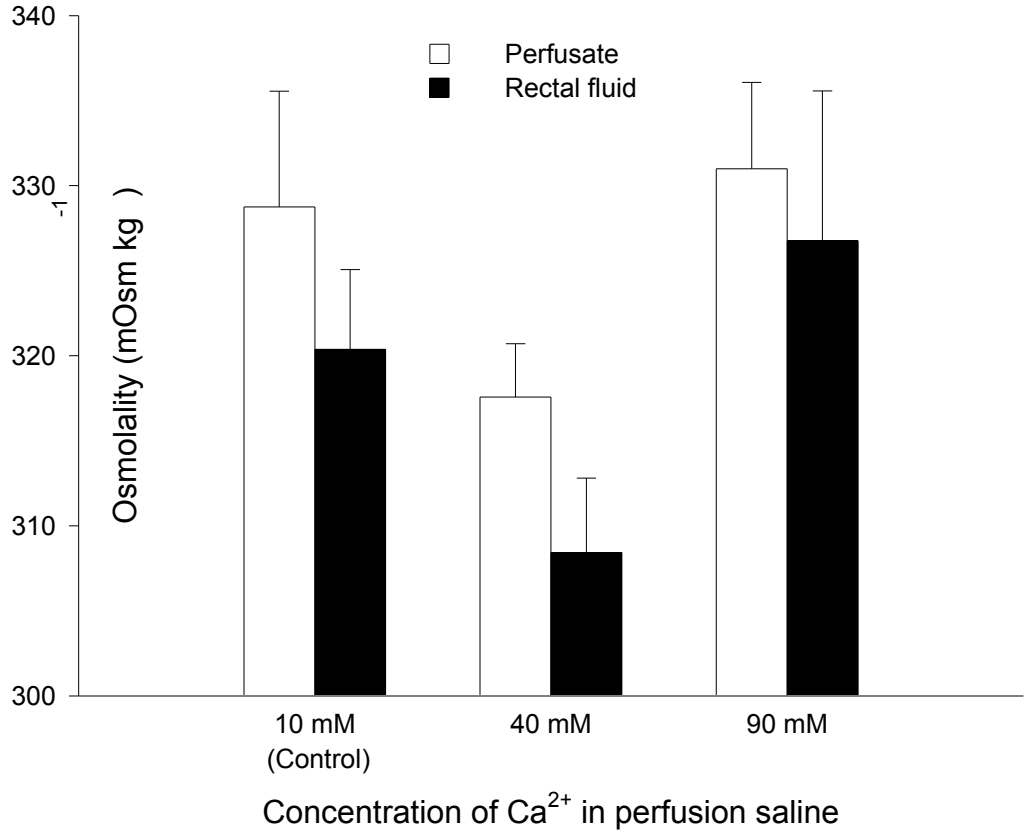



Figure 5.3: The mean (±SE) osmolality (mOsm $kg^{-1}$) of the perfusate entering the intestine and the voided rectal fluid of the flounder following perfusion with varying concentrations of calcium, 10 mM (control), 40 mM and 90 mM for 3 days. The open and shaded bars represent the osmotic pressure of the perfusate (prior to entering the intestine) and rectal fluid, respectively (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively).

absorption were considered reliable as the mean rate of intestinal perfusion was consistent across treatments (4.91 ±0.36, 4.67 ±0.21 and 5.50 ±0.57 ml $kg^{-1}$ $h^{-1}$ for control, 40 mM and 90 mM perfusion salines, respectively). This was further indicated after finding that fluid absorption by the intestine was independent of perfusion rate for each treatment (Control, R = 0.143, P = 0.735; 40 mM $Ca^{2+}$, R = -0.041, P = 0.931 and 90 mM $Ca^{2+}$, R = 0.011, P = 0.980).

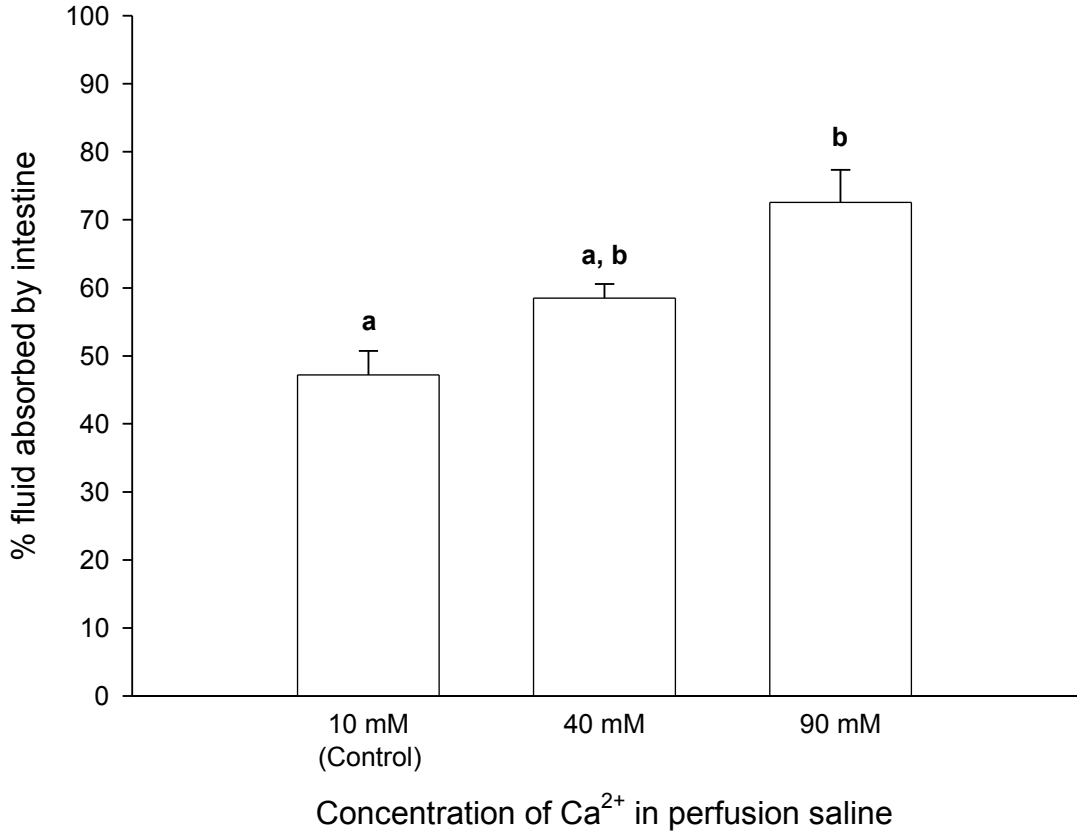



Figure 5.4: The mean (±SE) proportion of fluid absorbed by the flounder intestine following perfusion with varying concentrations of calcium, 10 mM (Control), 40 mM, 90 mM for 3 days (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively.

### 4.4 Fate of the divalent cations ($Ca^{2+}$ and $Mg^{2+}$)

The rate at which $Ca^{2+}$ was incorporated into carbonate precipitates correlated well with the amount perfused into the intestine. While the amount recovered in the rectal and intestinal fluid was low for both the control and 40 mM treatment, it was much higher at 90 mM $Ca^{2+}$. When the recoveries from these respective compartments (fluid + precipitates) are stacked together as in Figure 5.5A, they show that across treatments there was also an increasing amount of $Ca^{2+}$ unaccounted for (i.e. not showing up in either the fluid or precipitates).

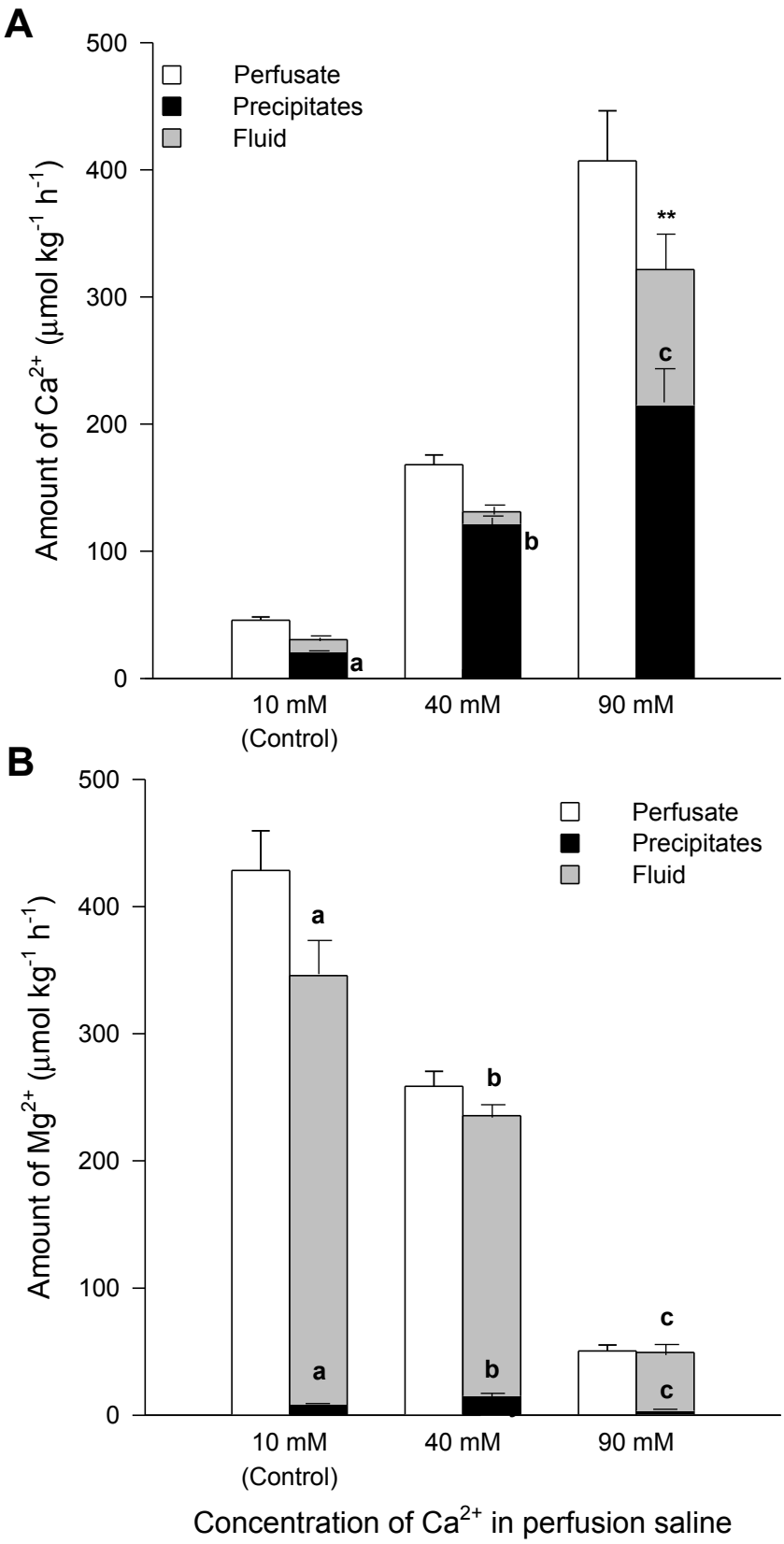

A
Perfusate
Precipitates
Fluid
Amount of Ca2+ (µmol kg-1 h-1)
500
400
300
200
100
0
a
b
c
**
10 mM
(Control)
40 mM
90 mM
B
Perfusate
Precipitates
Fluid
Amount of Mg2+ (µmol kg-1 h-1)
500
400
300
200
100
0
a
a
b
b
c
c
10 mM
(Control)
40 mM
90 mM
Concentration of Ca2+ in perfusion saline

Figure 5.5: The mean (±SE) amounts of calcium (panel A) and magnesium (panel B) (µmol $kg^{-1}$ $h^{-1}$) presented to, and recovered from the intestine and rectal catheters of the flounder following perfusion of the intestine with varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM for 3 days. The asterisks (**) represent a significant difference from the two corresponding treatments ($P < 0.001$) and means labelled with different letters indicate a significant difference ($P < 0.05$), n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively.

The rate of $Mg^{2+}$ being perfused into the intestine varied inversely with $Ca^{2+}$, since in the design of the perfusion saline $Mg^{2+}$ was substituted to permit increases in $Ca^{2+}$ concentration (in the form of their respective chloride salts) while maintaining $Cl^-$ and osmolality (Figure 5.5B). In relation to $Ca^{2+}$ the majority of $Mg^{2+}$ remained within the intestinal fluid, and very little was incorporated into the precipitates although this did vary significantly between treatments and precipitates recovered from fish undergoing the 40 mM treatment contained 45 and 79 % more $Mg^{2+}$ than the control or 90 mM treatments, respectively.

**4.5 Blood chemistry**

Compared with corresponding samples taken on Day 1, by the final day of perfusion (Day 3) the osmotic pressure of the plasma for fish receiving the 40 mM and 90 mM treatments was almost identical, although it had fallen by an average of 12 mOsm $kg^{-1}$ in the former treatment and only 6 mOsm $kg^{-1}$ in the latter. In contrast, for fish undergoing the control treatment plasma osmolality steadily rose over the 3 day perfusion period by an average of 9 mOsm $kg^{-1}$. Surprisingly, the measured pH and $TCO_2$ of plasma during perfusion with 90 mM $Ca^{2+}$ remained extremely stable, in comparison with individuals from other treatments where there appeared a tendency for pH to rise. In terms, of ion balance there was no evidence of disruption, particularly $Ca^{2+}$ which was tightly regulated by fish from all treatments (Table 5.3).

Table 5.3: A summary of the various acid-base and osmoregulatory parameters measured on whole blood (pH) and plasma (Osmolality, $TCO_2$, $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) as part of the daily blood sampling routine during perfusion of the flounder intestine with salines containing varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM over a 3 day period. Values represent mean (±SE). For each day means labelled with different letters are significantly different ($P<0.05$), n = 8, 7 and 8 for the Control, 40 mM and 90 mM treatments, respectively.

| | **Osmolality** (mOsm $kg^{-1}$) | **pH** | **$TCO_2$** (mM) | **$Na^+$** (mM) | **$Cl^-$** (mM) | **$K^+$** (mM) | **$Ca^{2+}$** (mM) | **$Mg^{2+}$** (mM) |
|---|---|---|---|---|---|---|---|---|
| **Day 1** | | | | | | | | |
| Control | 326 (5.6) | 7.88 (0.03) | 7.1 (0.3)$^{a}$ | 161.7 (3.6) | 147.1 (1.3) | 3.0 (0.1)$^{a,b}$ | 2.2 (0.2)$^{a}$ | 0.6 (0.1) |
| 40 mM | 330 (2.9) | 7.81 (0.03) | 5.7 (0.4)$^{b}$ | 163.8 (1.1) | 145.9 (1.1) | 2.7 (0.1)$^{b}$ | 2.8 (0.1)$^{b}$ | 0.4 (0.1) |
| 90 mM | 323 (2.9) | 7.78 (0.04) | 6.7 (0.3)$^{a,b}$ | 161.7 (2.6) | 148.0 (1.7) | 3.4 (0.2)$^{a}$ | 2.0 (0.2)$^{a}$ | 0.6 (0.2) |
| **Day 2** | | | | | | | | |
| Control | 329 (5.9) | 7.92 (0.02)$^{a}$ | 7.0 (0.4) | 159.2 (3.8) | 146.1 (1.6) | 3.0 (0.1)$^{a}$ | 2.0 (0.2) | 0.7 (0.1) |
| 40 mM | 323 (2.8) | 7.81 (0.03)$^{b}$ | 5.6 (0.4) | 156.7 (2.9) | 145.0 (1.3) | 2.6 (0.1)$^{b}$ | 2.4 (0.1) | 0.4 (0.1) |
| 90 mM | 324 (6.8) | 7.80 (0.05)$^{b}$ | 6.9 (0.4) | 158.6 (3.0) | 145.5 (1.6) | 3.1 (0.1)$^{a}$ | 1.9 (0.1) | 0.6 (0.3) |
| **Day 3** | | | | | | | | |
| Control | 335 (5.0)$^{a}$ | 7.94 (0.02)$^{a}$ | 5.8 (0.6) | 159.6 (4.2) | 148.4 (3.3) | 3.0 (0.1)$^{a,b}$ | 2.0 (0.1) | 0.9 (0.2) |
| 40 mM | 318 (2.6)$^{b}$ | 7.89 (0.02)$^{a,b}$ | 6.3 (0.5) | 160.7 (2.4) | 144.3 (1.1) | 2.6 (0.1)$^{b}$ | 2.4 (0.1) | 0.4 (0.1) |
| 90 mM | 317 (5.0)$^{b}$ | 7.81 (0.03)$^{b}$ | 6.9 (0.7) | 157.0 (3.4) | 146.8 (2.2) | 3.2 (0.1)$^{a}$ | 1.9 (0.1) | 1.6 (0.9) |

# 5. Discussion

The act of drinking by teleosts occupying a hyper-osmotic media such as seawater presents the gastrointestinal tract with a unique challenge. Of particular interest for this study was the role of $HCO_3^-$ secretion and precipitation by the intestine and consequences for fluid absorption and divalent ion homeostasis. Over the past two decades, various investigations into the phenomena of intestinal $HCO_3^-$ secretion have led to the suggestion that the precipitation of $CaCO_3$ will reduce luminal osmotic pressure and create a favourable osmotic gradient for additional fluid absorption (Humbert *et al*., 1986; Walsh *et al*., 1991; Wilson *et al*., 1996). From a series of elegant *in vivo* perfusion experiments Wilson *et al*. (2002) presented the first evidence for this novel mechanism of fluid transport. However, they were unable to demonstrate a significant increase in fluid absorption following perfusion of the intestine with saline containing 20 mM $Ca^{2+}$ used to stimulate $HCO_3^-$ secretion and precipitation. The present study employed the same *in vivo* techniques but applied even higher concentrations of $Ca^{2+}$ (40 mM and 90 mM). In terms of stimulating $HCO_3^-$ production and precipitation the results fit in well with, and corroborate, the prior observations made by Wilson *et al*. (2002). At 40 mM $Ca^{2+}$ the amount of carbonate precipitates increased more than 4-fold (Figure 5.2), but fluid absorption was only increased by a modest 11 % (Figure 5.4) compared with the control treatment. However, at 90 mM $Ca^{2+}$, not only was the rate of precipitation even higher, but fluid absorption had increased significantly also, by ~50 % over the controls.

### 5.1 The influence of luminal osmotic pressure

The hypothesis behind the role of precipitation in fluid transport is that the alkaline conditions created by $HCO_3^-$ secretion will promote the precipitation of $Ca^{2+}$, and to a lesser extent $Mg^{2+}$, to their respective carbonates. By removing the osmotic influence of these ions this will effectively reduce the osmolality within the gut lumen consequently providing an additional, osmotic driving force for fluid absorption (Wilson *et al*., 2002). It would therefore be logical to assume that at higher rates of precipitation the osmotic pressure of the rectal fluid would be lower. However, despite the vastly higher rates of precipitation in the 40 mM and 90 mM treatments this did not lead to concomitant reductions in rectal fluid osmolality (Figure 5.2). The reason for this is not clear but may be due to the ion species

left behind in the rectal fluid. In an attempt to avoid problems of spontaneous precipitation of $CaSO_4$, levels of $SO_4^{2-}$ in the perfusion salines were reduced to 10 mM (Table 5.1), effectively being replaced by $Cl^-$, in the form of $CaCl_2$ and $MgCl_2$. As a consequence, the excreted rectal fluid was high in $MgCl_2$ for the control and 40 mM treatments, and a combination of $CaCl_2$ and NaCl at 90 mM (Table 5.4). All of these chloride salts possess relatively high osmotic coefficients (0.86, 0.89 and 0.93, respectively, Robinson and Stokes, 1965), whereas the coefficient for $MgSO_4$, which typically dominates in the excreted rectal fluid, is much lower (0.58) and would therefore

Table 5.4: The ionic composition of the rectal fluid following intestinal perfusion with varying concentrations of calcium, 10 mM (Control), 40 mM, 90 mM for 3 days (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively). Values are given as mean ±SE and presented as mM or mEq where appropriate and osmolality measured as mOsm $kg^{-1}$. Means labelled with different letters indicate a significant difference ($P < 0.05$), n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively.

| Ion | Perfusion treatment | | |
|---|---|---|---|
| | Control | 40 mM $Ca^{2+}$ | 90 mM $Ca^{2+}$ |
| $Na^+$ | 16.9 ±1.8[a] | 19.0 ±3.2[a] | 62.8 ±18.4[b] |
| $Cl^-$ | 162.4 ±7.7 | 163.4 ±6.2 | 178.1 ±8.8 |
| $K^+$ | 0.6 ±0.2[a] | 0.5 ±0.1[a] | 2.2 ±0.4[b] |
| $Ca^{2+}$ | 3.7 ±1.0[a] | 4.3 ±1.1[a] | 67.1 ±6.5[b] |
| $Mg^{2+}$ | 131.4 ±3.9[a] | 115.9 ±5.0[a] | 41.1 ±7.8[b] |
| $SO_4^{2-}$* | 19.1 ±1.9 | - | 42.3 ±10.0 |
| $HCO_3^- + CO_3^{2-}$ | 68.4 ±5.6[a] | 37.3 ±7.3[b] | 6.6 ±1.3[c] |
| Osmolality | 320 ±5 | 308 ±4 | 327 ±9 |

*n = 5 (Control) and n = 7 (90 mM). Samples from the 40 mM treatment are still to be analysed.

translate to a much lower osmotic pressure. Alternatively, this analysis does not take into account localised osmotic gradients created by $CaCO_3$ precipitation which are likely to be driving this additional absorption of fluid and therefore differences in rectal fluid osmolality would not have been detected here as measurements were made on bulk fluid samples only.

**5.2 Solute-linked water transport**

In order to produce the higher rates of fluid absorption shown in Figure 5.4, the importance of carbonate precipitation cannot be under-stated, but the advantage to osmotically driven fluid transport does not appear obvious in relation to the osmotic pressure of the rectal fluid as predicted. Current understanding of the mechanism of fluid uptake by the teleost intestine (in terms of osmoregulation) involves the process of "solute-linked water transport", which is principally associated with the active transport of $Na^+$ and $Cl^-$ across the apical membrane thus establishing the osmotic gradient along

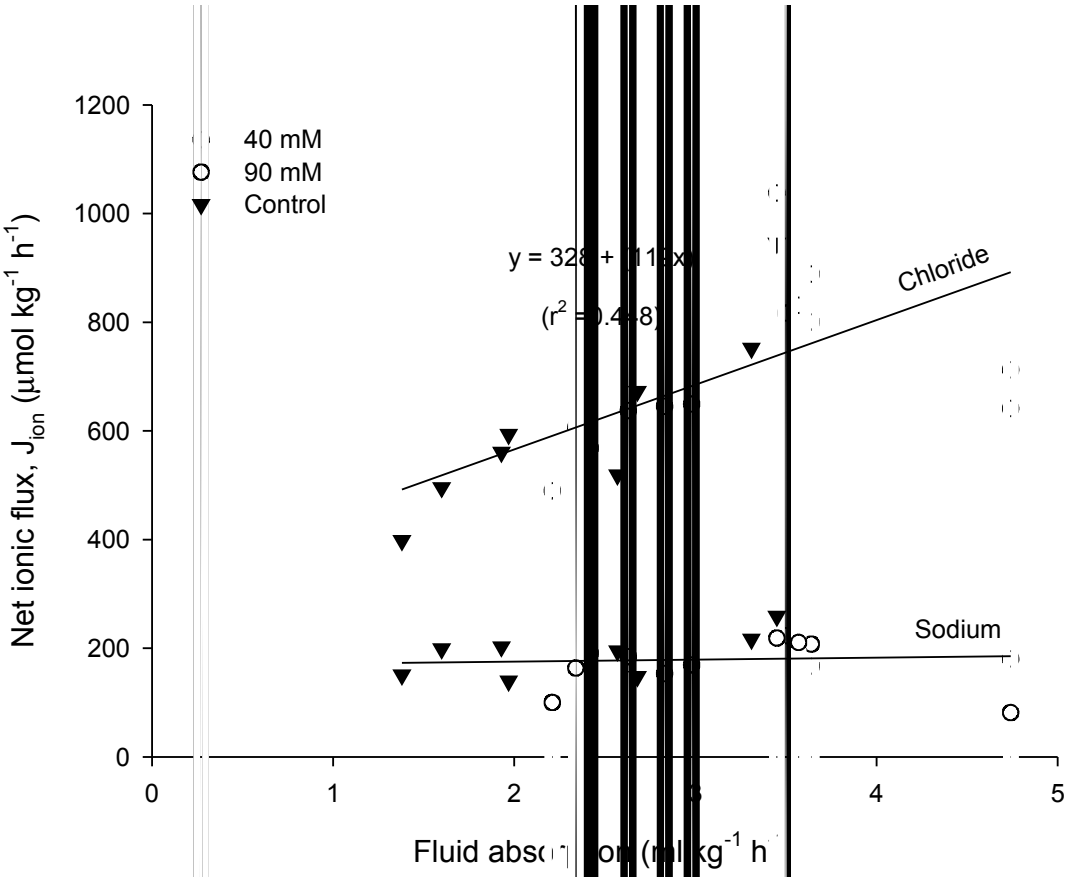



Figure 5.6: The relationship between the net fluxes of sodium and chloride (µmol $kg^{-1}$ $h^{-1}$) and the corresponding rate of fluid absorption (ml $kg^{-1}$ $h^{-1}$) by the intestine of the flounder perfused with salines containing varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM over a total of 3 days (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively).

which water passively follows into the body. Examining the rate of fluid absorption in relation to the net fluxes of $Na^+$ and $Cl^-$ across treatments revealed an interesting trend,

Figure 5.6 clearly shows that increases in fluid transport were completely independent of changes in $Na^+$ absorption ($F_{1, 21} = 0.14$, $P = 0.771$) but displayed a close, positive relationship with $Cl^-$ absorption ($F_{1, 21} = 17.07$, $P = <0.001$). It was therefore possible to confidently rule out any changes to NaCl cotransport being involved in this additional fluid absorption, along with any potential influence of perfusion rate (Section 4.3).

### 5.3 A role for apical $Cl^-/HCO_3^-$ exchange

A prominent role for $Cl^-$ was not surprising given the significant increases in total $HCO_3^-$ equivalent secretion between treatments (Figure 5.2) and the proposed involvement of an apical $Cl^-/HCO_3^-$ exchanger (Grosell and Jensen, 1999; Grosell *et al.*, 2005; Grosell, 2006), which in itself is capable of driving $Cl^-$ and fluid absorption, as demonstrated *in vitro* (Grosell *et al.*, 2005). Even though average net $Cl^-$ absorption did not significantly increase following perfusion with 90 mM $Ca^{2+}$ (Figure 5.7), making it difficult to directly relate to $Cl^-/HCO_3^-$ exchange, a significant positive relationship was present between $Cl^-$ absorption and $HCO_3^-$ secretion (Pearson correlation, $R = 0.466$, $P = 0.025$, $n = 23$). In spite of this, $Cl^-$ absorption greatly exceeded the sum of all the other corresponding cation fluxes ($Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) by an average of 50 % (Figure 5.7). Since charge balance must be observed in the absorbed fluid, this indicates a substantial shortfall in absorbed cations (or secreted anions). It should be noted that $SO_4^{2-}$, which was the only other anion present in the perfusion saline, has not been included since only a limited number of samples have been analysed so far. Although as expected, the net $SO_4^{2-}$ flux is currently not significantly different from zero (control: 2.8 ±4.4 µmol $kg^{-1}$ $h^{-1}$, $n = 5$ and 90 mM $Ca^{2+}$: 5.5 ±6.1 µmol $kg^{-1}$ $h^{-1}$, $n = 7$), hence the shortfall is likely to be uptake of a 'missing' cation.

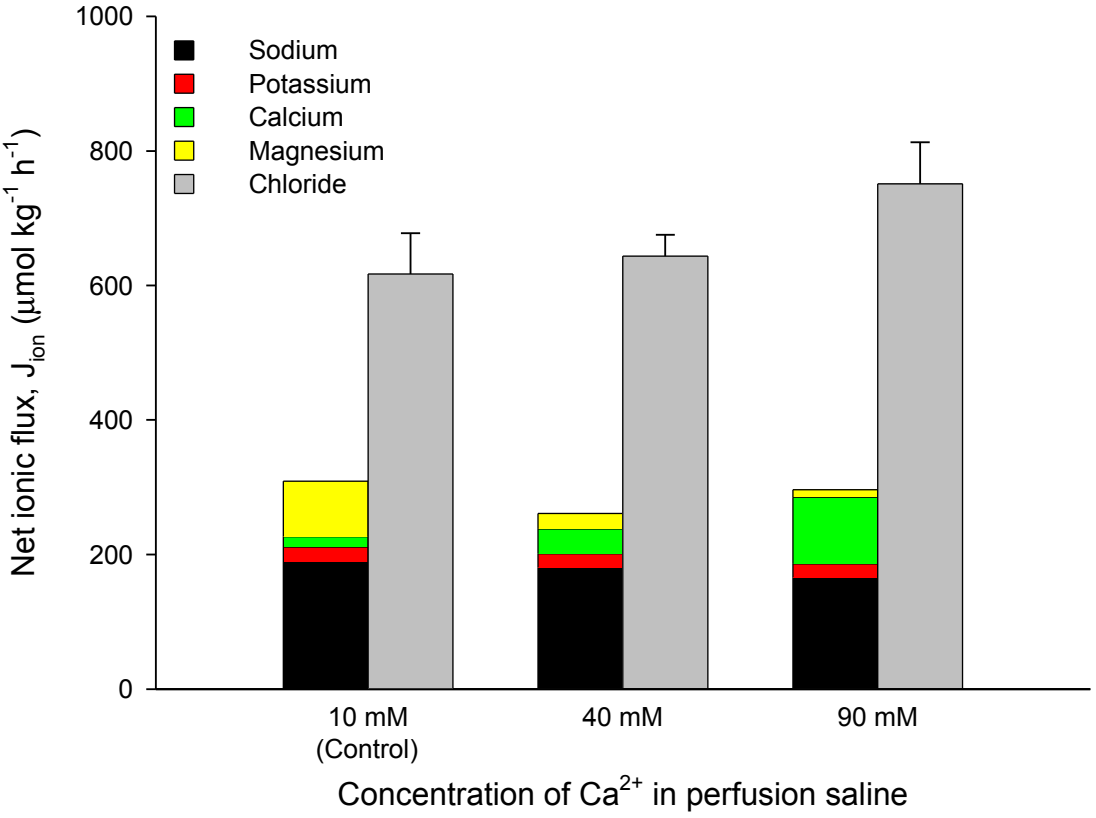



Figure 5.7: A comparison of the mean (±SE) net fluxes of cations and anions (µmol $kg^{-1}$ $h^{-1}$) by the intestine of the flounder following perfusion with salines containing varying concentrations of calcium, control (10 mM), 40 mM and 90 mM (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively).

### 5.4 Endogenous $CO_2$ hydration

A likely candidate for this cation imbalance is $H^+$, being derived from intracellular $CO_2$ hydration which is fuelling $HCO_3^-$ secretion. For the flounder, which relies largely on this form of endogenous $HCO_3^-$ production (Wilson and Grosell, 2003), it is important that there is effective removal of $H^+$, preferably across the basolateral membrane, allowing accumulation of $HCO_3^-$ for apical anion exchange while preventing cellular acidification and reversal of the hydration reaction (Grosell *et al.*, 2001; Grosell *et al.*, 2005; Grosell, 2006). The polarity of these acid-base transfers by the intestine have subsequently been demonstrated on isolated preparations from the flounder (Chapter 3, Section 4.3), as well as

the toadfish (Grosell and Genz, 2006; Grosell and Taylor, 2007) and killifish (Chapter 6, Section 4.2.3). Since $CO_2$ exerts a negligible osmotic pressure, the generation of $HCO_3^-$ and $H^+$ and subsequent apical secretion of $HCO_3^-$ in exchange for $Cl^-$ will lead to a net gain of osmolytes by the cell (Wilson *et al.*, 2002), hence $H^+$ is likely to form part of the net solute flux, contributing to water transport (Grosell, 2006) and osmolarity of the absorbed fluid (Grosell and Taylor, 2007). Thus, assuming the missing cation was indeed $H^+$, then it comes as no surprise that the rate of '$H^+$ absorption' was almost directly proportional to the corresponding rate of total (Fluid + Precipitates) $HCO_3^-$ secretion by the intestine for each individual across all treatments (Figure 5.8).

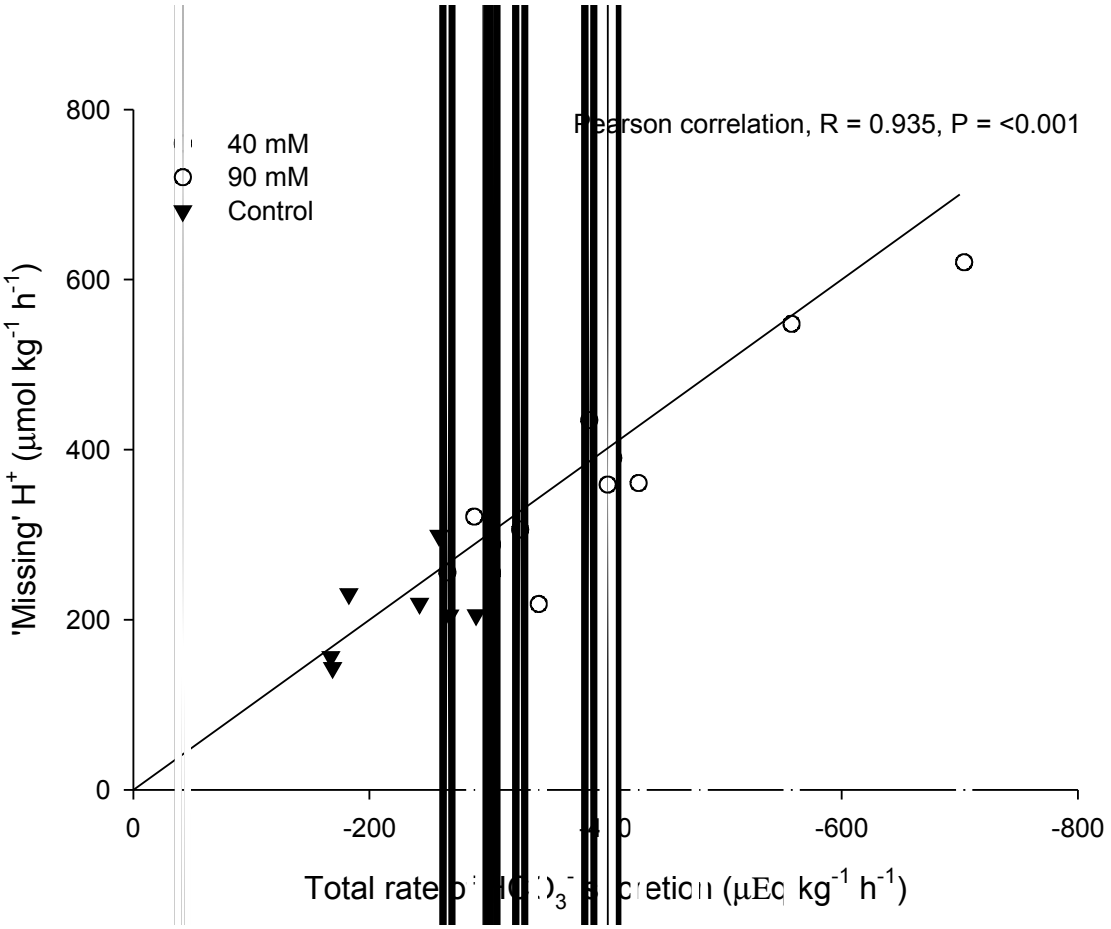



Figure 5.8: The relationship between the total rate of $HCO_3^-$ secretion (µEq $kg^{-1}$ $h^{-1}$) and 'missing cation', presumed to be $H^+$ (µmol $kg^{-1}$ $h^{-1}$) absorbed by the intestine of the flounder perfused with salines containing varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM over a total of 3 days (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively).

Interestingly, this relationship persists across all treatments even at 90 mM $Ca^{2+}$ which would appear contrary to the prediction that up-regulation of $HCO_3^-$ secretion will draw upon exogenous $CO_2$ stores. After increasing the ambient $Ca^{2+}$ concentration of the surrounding seawater to 40 and 70 mM, Wilson and Grosell (2003) found a significant reduction in plasma total $CO_2$ after 72 hours. In the present study, although plasma $TCO_2$ was significantly reduced by 20 % after 24 hours at 40 mM $Ca^{2+}$ compared with the controls, overall there was no marked difference between the control and 90 mM treatments, and indeed across all treatments by day 3 of perfusion (Table 5.3) which would support the relationship displayed in Figure 5.8, that endogenous $CO_2$ is sufficient to fuel the increase in $HCO_3^-$ secretion observed over these three experimental conditions.

### 5.4.1 Intestinal $H^+$ production and systemic acid-base balance

From a whole animal perspective, while intestinal $HCO_3^-$ secretion does not have a role in systemic acid-base regulation *per se*, it does represent a significant base efflux that is balanced by an equivalent net acid efflux (or base uptake) across the gills (Wilson *et al*., 1996; Wilson and Grosell, 2003). The identity of this missing cation as $H^+$ can be further argued for when the net flux of the 'missing cation' is compared with the net (extra-intestinal) flux of titratable acid (the reverse of measured titratable alkalinity) into the surrounding seawater (from Cooper, C. A., Whittamore, J. M. and Wilson, R. W., in preparation), again displaying a strong, significant correlation (Figure 5.9). Furthermore, the blood pH of fish where the intestine was being perfused with higher concentrations of $Ca^{2+}$, and consequently producing more $HCO_3^-$, was 0.07 to 0.10 pH units lower than controls on day one, and for the 90 mM treatment remained significantly lower over subsequent days (Table 5.3). These observations would also appear consistent with the circulation bearing an increased burden of acid at higher rates of $HCO_3^-$ secretion. Most striking is that the relationships with total $HCO_3^-$ secretion shown in Figures 5.8 and 5.9 are between data that were collected independently from each fish (i.e. intestinal ion fluxes and gill fluxes, respectively).

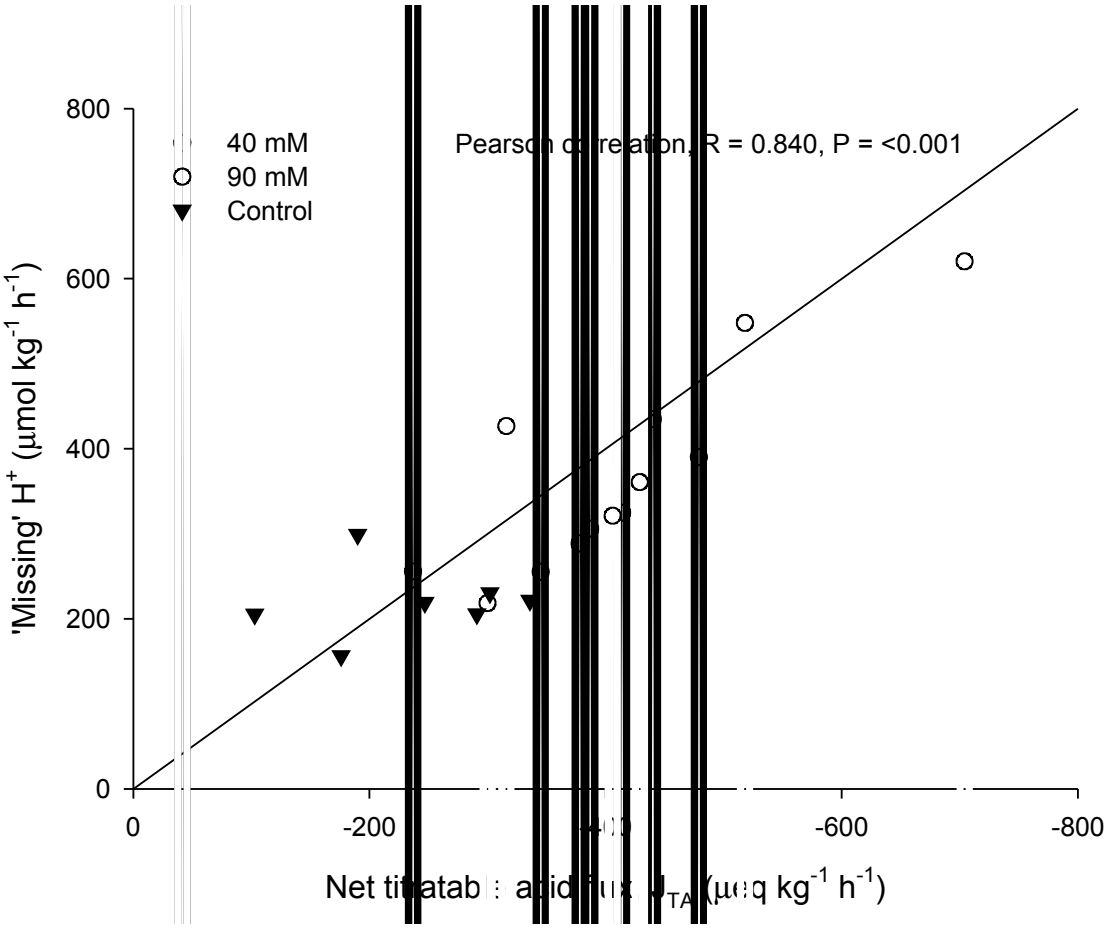



Figure 5.9: The relationship between the net titratable acid flux (µEq $kg^{-1}$ $h^{-1}$) *via* non-intestinal routes (i.e. gills or kidney) into the surrounding seawater and the 'missing cation', presumed to be $H^+$ (µmol $kg^{-1}$ $h^{-1}$) absorbed by the intestine of the flounder perfused with saline containing varying concentrations of calcium, 10 mM (Control), 40 mM and 90 mM over a total of 3 days (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively).

### 5.5 The contribution of $HCO_3^-$ production, secretion and precipitation to intestinal fluid absorption

Along with carbonate precipitation there is also the net gain of osmolytes ($H^+$ and $Cl^-$) from $HCO_3^-$ production and secretion that can potentially drive fluid absorption in the absence of a net increase in NaCl. The transport of NaCl involves NaCl cotransport in parallel with $Na^+$-$K^+$-$2Cl^-$ cotransport, thus with calculations of the net solute fluxes at hand an attempt was made to define the relative contributions of NaCl-driven, solute-linked water transport from the process of $HCO_3^-$ secretion and precipitation for these calculations. The sum of the

net fluxes of $Na^+$ and $K^+$ were therefore considered to be associated with an equal net flux of $Cl^-$. By taking the molarity of water as 55.56 M and assuming that the associated fluid being absorbed under these conditions was iso-osmotic to the perfusion saline the stoichiometric relationship between water and solutes in the absorbed fluid was calculated as 170 moles water: 1 mole solute (at an osmolality of 326 mOsm $kg^{-1}$). Using this information it was possible to calculate the theoretical rate of

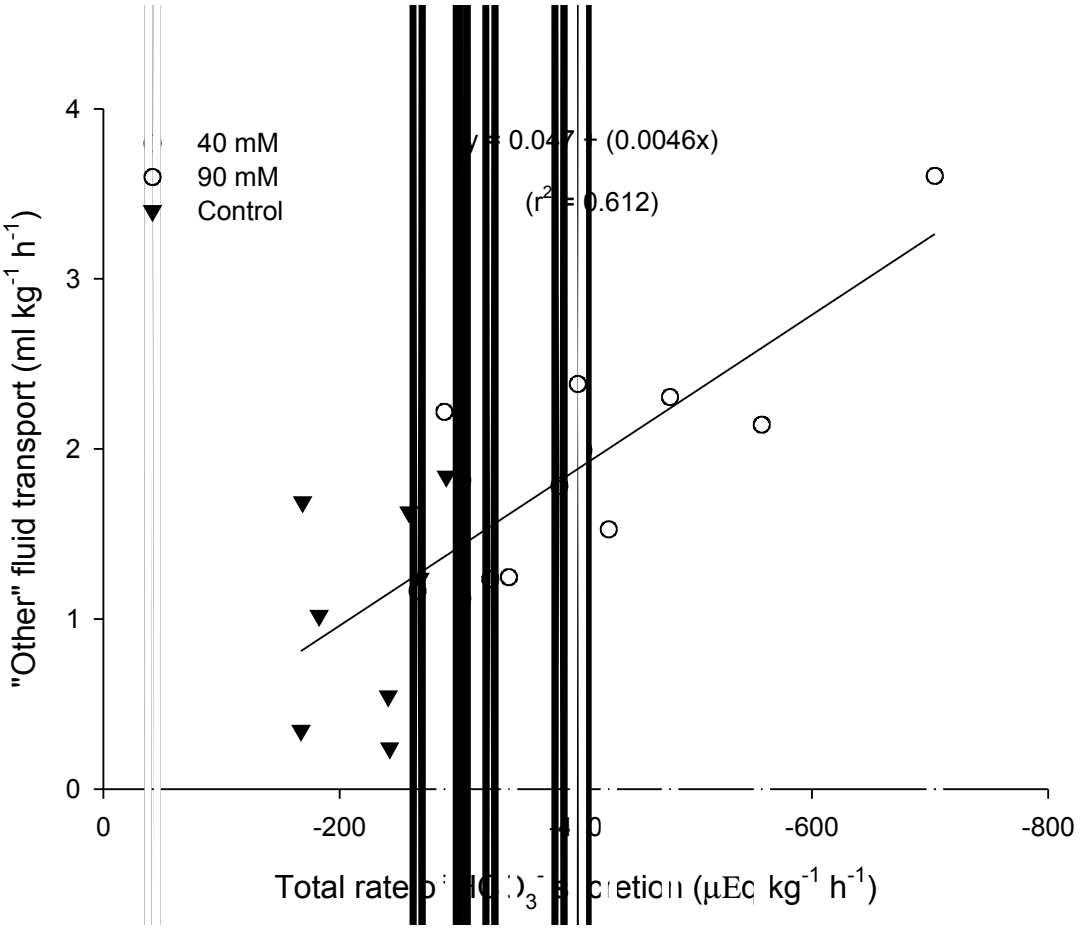



Figure 5.10: The relationship between the rate of fluid transport (ml $kg^{-1}$ $h^{-1}$) predicted to be associated with $HCO_3^-$ production, secretion and precipitation, and the total rate of $HCO_3^-$ secretion (µEq $kg^{-1}$ $h^{-1}$) by the intestine of the flounder following perfusion with varying concentrations of calcium, control (10 mM), 40 mM and 90 mM over a total of 3 days (n = 8, 7 and 8 for the control, 40 mM and 90 mM treatments, respectively).

fluid absorbed per kg body weight (ml $kg^{-1}$ $h^{-1}$) associated with $Na^+$, $K^+$ and $Cl^-$ (i.e. solute-linked water transport). By subtracting this value from the actual measured rate of fluid absorption it was possible to derive the theoretical contribution made by $HCO_3^-$ production,

secretion and precipitation to fluid transport. Interestingly, there proved to be a significant relationship between this rate of fluid absorption and total $HCO_3^-$ secretion (Figure 5.10).

### 5.5.1 A hypo- or hyperosmotic absorbate?

Curiously, the slope of the regression shown in Figure 5.10 equates to 254 moles water absorbed per mole $HCO_3^-$ secreted, and compared to the iso-osmotic relationship quoted above (170 moles water: 1 mole solute) suggests that the fluid absorbed in association with $HCO_3^-$ would be distinctly hypo-osmotic (~219 mOsm $kg^{-1}$) compared with the perfusion saline (~326 mOsm $kg^{-1}$). However, based on the net fluxes of all measured ions ($Na^+$, $K^+$, $Cl^-$, $Ca^{2+}$, $Mg^{2+}$ and $H^+$) from the perfusion saline, and assuming an overall osmotic coefficient of 0.9, the osmolarity of the fluid absorbed from the intestine was in fact the opposite and distinctly hyper-osmotic with calculated osmolarities of 448 ±25, 406 ±9 and 388 ±13 mOsm $l^{-1}$ for the control, 40 mM and 90 mM treatments, respectively.

There are a number of possibilities that can account for the progressive reduction in osmotic pressure of fluids along the intestine and general hypo-osmolarity of the rectal fluid in relation to blood plasma. Firstly, this is the result of $CaCO_3$ precipitation, reducing the osmotic influence of $Ca^{2+}$ in the lumen (Wilson *et al.*, 2002). Secondly, following absorption of the majority of NaCl this leaves behind a fluid rich in $MgSO_4$ which has a lower osmotic coefficient and therefore exerts a reduced osmotic pressure (Taylor and Grosell, 2006b). One further (and somewhat controversial) suggestion is the absorption of a hyper-osmotic fluid from the intestine which will also assist this reduction in osmotic pressure (Shehadeh and Gordon, 1969; Skadhauge, 1974; Grosell, 2006; Grosell and Taylor, 2007). This would be in marked contrast to the iso-osmotic, or near iso-osmotic, fluid absorbed by other vertebrate epithelia (Curran, 1960; Diamond, 1964; Spring, 1999; Larsen *et al.*, 2002). A number of *in vivo* and *in vitro* studies have apparently demonstrated this latter phenomenon of a hyper-osmotic absorbate. For example, the fluid absorbed following perfusion of the European eel (*Anguilla anguilla*) intestine with diluted seawater was hyper-osmotic to the blood plasma (Skadhauge 1969; 1974). Similarly, early *in vitro* experiments by House and Green (1965) using short-horned sculpin (*Cottus scorpius*) and a more recent review spanning a range of species, including the flounder (Grosell, 2006), have all indicated a hyperosmotic absorbate.

Using gut sac preparations from the toadfish (*Opsanus beta*), Grosell and Taylor (2007) have shown that in the absence of an osmotic gradient and under near symmetrical conditions (i.e. high NaCl, low $MgSO_4$), the absorbed fluid was iso-osmotic (299 mOsm $l^{-1}$) similar to other fluid transporting epithelia. Yet, when presented with *in vivo*-like conditions (low NaCl, high $MgSO_4$) the absorbate was hyper-osmotic (380 mOsm $l^{-1}$). It was predicted that luminal osmotic pressure was higher due to the elevated $HCO_3^-$ secretion under the latter conditions which incurred higher rates of $Cl^-$ and $H^+$ absorption (arising from anion exchange and $CO_2$ hydration, respectively). However, these observations and those mentioned previously are all based on ion fluxes in relation to the mucosal saline, and as pointed out by Grosell and Taylor (2007) do not represent actual conditions across the epithelia. It is particularly important to bear this point in mind because if the intestine were actually absorbing an increasingly hyper-osmotic fluid in relation to elevated $HCO_3^-$ secretion this would directly contradict earlier work by Wilson *et al.* (2002) who found a significant reduction in osmotic pressure of the blood plasma by 7 mOsm $kg^{-1}$ associated with elevated intestinal $HCO_3^-$ secretion rates. Indeed, for the present study, blood plasma had been reduced 18 mOsm $kg^{-1}$ by day 3 of perfusion with 40 and 90 mM $Ca^{2+}$, compared with the control (Table 5.3) which is consistent with the prediction of a hypoosmotic absorbate (Figure 5.10). The question therefore becomes how is it possible to absorb a hyperosmotic fluid from the intestine but actually reduce the osmotic pressure of the surrounding body fluids?

### 5.5.2 A role for $CO_2$ in intestinal fluid absorption

Given the increasing proportion of $H^+$ in the absorbed fluid, from 24 % in controls up to 40 % at 90 mM $Ca^{2+}$, inferred from Figure 5.8, such an acidic fluid (pH <1) would need to be buffered. Therefore, one way to achieve a hypoosmotic absorbate would be the conversion of $H^+$ into $CO_2$ in the interstitial fluid before entering the portal vein where it would be transported in the blood and removed at the gills (Figure 5.11). This is similar, in principal, to how the mammalian duodenum neutralises large amounts of gastric acid, by secreting $HCO_3^-$ into the lumen and absorbing the resulting $CO_2$ into the body (Mizumori *et al.*, 2006). In addition to the presence of increasing rates of $H^+$ (produced endogenously during the formation of $HCO_3^-$) which were subsequently excreted into the surrounding seawater in association with elevated luminal $Ca^{2+}$ (indicated by Figures 5.7-5.9), observations of

blood acid-base parameters also lend some support to this hypothesis. Blood pH was typically reduced by more than 0.1 pH units in the high $Ca^{2+}$ treatments (Table 5.3), indicating an increased burden of acid, and along with a significant elevation of plasma $PCO_2$ in fish perfused with 90 mM $Ca^{2+}$ (Cooper, C. A., Whittamore, J. M. and Wilson, R. W., in preparation), would be consistent with increased amounts of $CO_2$ entering the bloodstream.

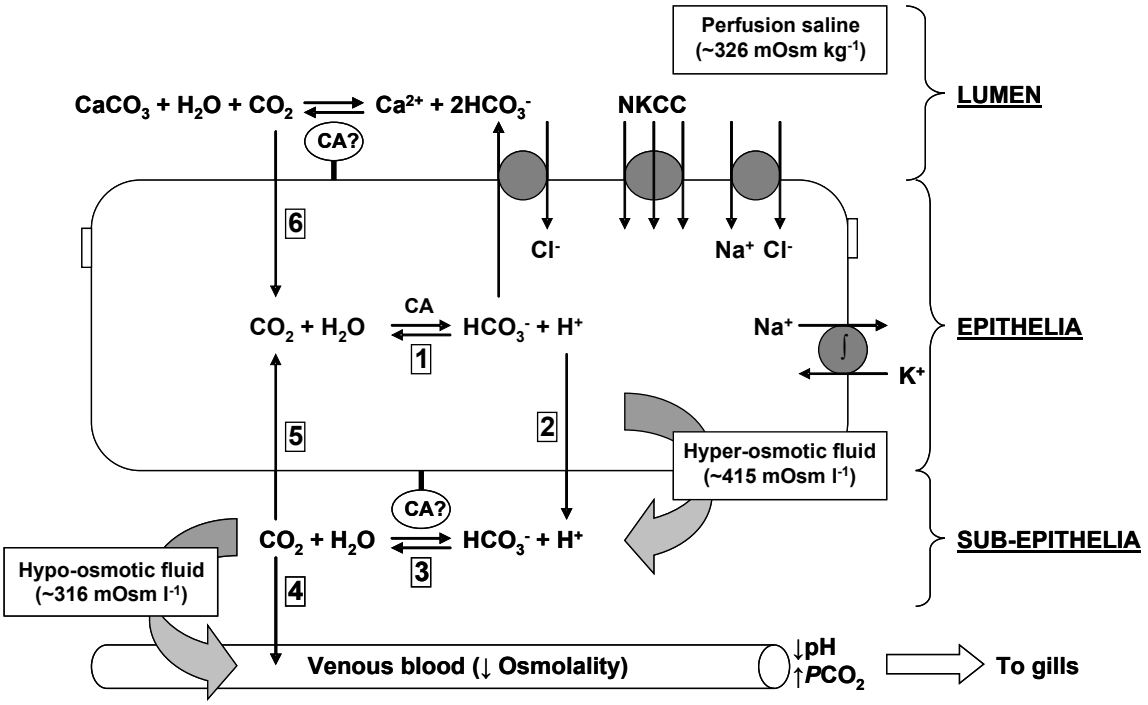



Figure 5.11: A proposed model illustrating the potential pathway for the removal of excess $H^+$ arising from intracellular $CO_2$ hydration (1). The resulting $H^+$ will be preferentially extruded across the basolateral membrane (2) as part of the hyper-osmotic absorbate. In the sub-epithelial compartment $H^+$ will be buffered by $HCO_3^-$ producing $CO_2$ which may well involve an extracellular carbonic anhydrase, CA? (3). This reaction will effectively remove the osmotic influence of these ions in the sub-epithelia and contribute to a substantial reduction in osmotic pressure within this compartment. The resulting $CO_2$ can then enter the venous blood (4) where it will have the effect of reducing pH and increasing $PCO_2$ before being removed at the gills. This $CO_2$ could also potentially recycle back across the basolateral membrane into the cell (5) to support further $HCO_3^-$ production. In addition, $HCO_3^-$ consumed in the production of $CaCO_3$ will also yield $CO_2$ which could similarly recycle back into the cell (6).

The conversion of $H^+$ into $CO_2$ would not only consume $H^+$ in the absorbed fluid but also $HCO_3^-$ in the sub-epithelial interstitium, and since $CO_2$ will exert a negligible osmotic effect will offer a considerable osmotic gain in terms of fluid absorption. With greater proportions of $H^+$ expected in the absorbed fluid at higher rates of $HCO_3^-$ secretion (Figures 5.7-5.9), this could conceivably produce the increasingly hypoosmotic absorbate predicted from Figure 5.10 and lead to the observed reduction in osmolality of the blood plasma (Table 5.3). Interestingly, if all of the $H^+$ in the absorbed fluid were removed as $CO_2$ into the bloodstream (or recycled back into the cell), then as predicted by Figure 5.11, this does indeed produce a hypoosmotic absorbate, becoming increasingly so at higher rates of intestinal $HCO_3^-$ secretion and precipitation as shown in Figure 5.12 ($F_{1, 20} = 23.68$, $P = <0.001$).

As shown by the model in Figure 5.11 there is also a link with $CaCO_3$ precipitation and the fate of its by-products, namely $H^+$ (in the form of $CO_2$) which can recycle back into the cell. This supposition is corroborated by observations that the process of $CaCO_3$ crystal formation takes place very close to the apical membrane, from surveys by electron microscopy (Humbert *et al.*, 1986; 1989), and could therefore involve an external, membrane bound carbonic anhydrase based on evidence gathered from the eel (Maffia *et al.*, 1996) and rainbow trout (Grosell *et al.*, 2007). In fact, as there are up to 3 potential sources of $CO_2$ fuelling $HCO_3^-$ secretion: (1) cellular metabolism, (2) dehydration of $HCO_3^-$ in the sub-epithelia and (3) $CaCO_3$ precipitation in the gut lumen, this model may also explain why $HCO_3^-$ production appeared to be almost exclusively derived from intracellular $CO_2$ hydration, even at the highest rates of secretion (Table 5.3; Figures 5.8

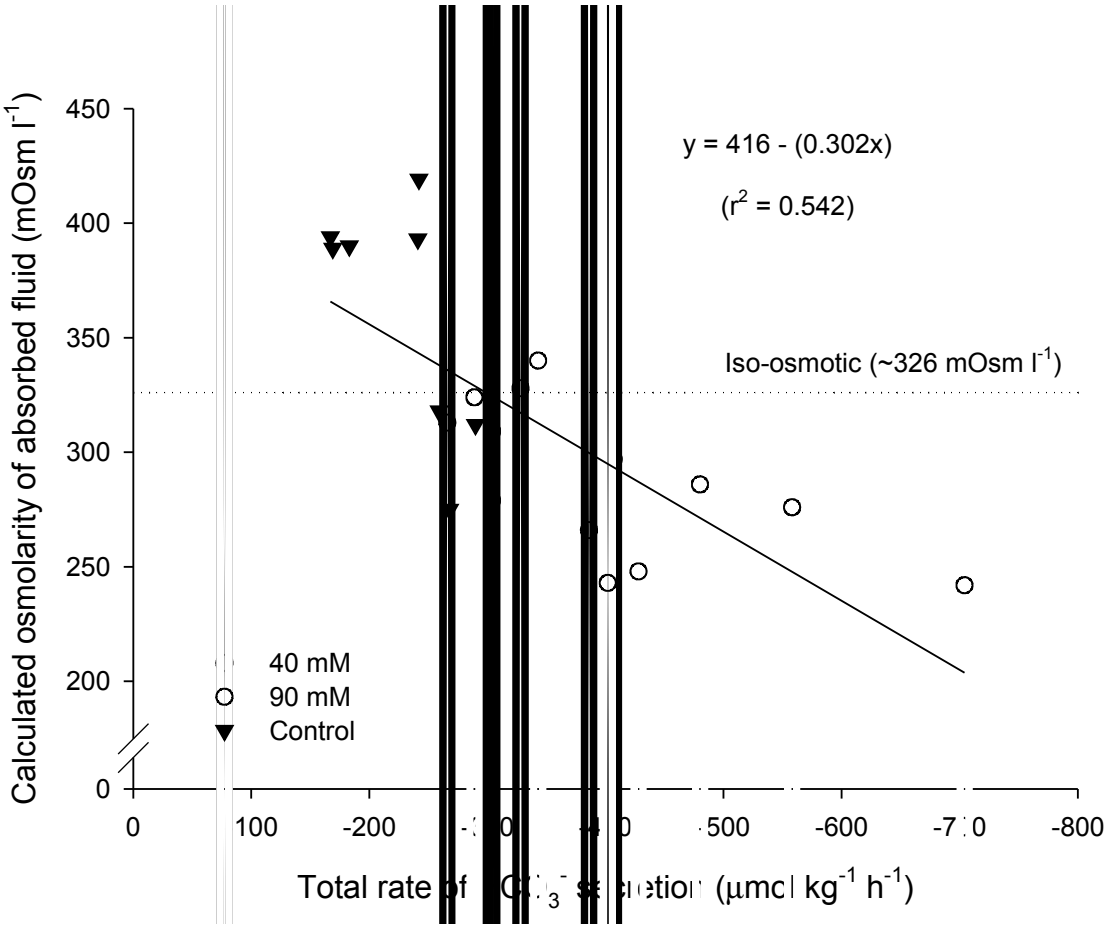



Figure 5.12: The relationship between calculated osmolarity of the absorbed fluid (mOsm $l^{-1}$), minus the contribution of $H^+$, and the measured total rate of $HCO_3^-$ secretion (µmol $kg^{-1}$ $h^{-1}$) by the intestine of the flounder following perfusion with varying concentrations of calcium, control (10 mM), 40 mM and 90 mM over a total of 3 days (n = 8, 7 and 7 for the control, 40 mM and 90 mM treatments, respectively).

and 5.9), even though it has been predicted that a high demand for intestinal $HCO_3^-$ secretion would be likely to draw upon exogenous sources of $CO_2$ in the plasma (Wilson and Grosell, 2003). In summary, analysis of the data from the present study has provided further evidence for the participation of $HCO_3^-$ production, secretion and $CaCO_3$ precipitation in fluid absorption by the marine teleost intestine, and highlighted some of the underlying intricacies behind this novel mechanism of water transport. The potential involvement of $CO_2$ moving between the lumen, epithelia and blood, supporting intestinal $HCO_3^-$ secretion, $CaCO_3$ precipitation and fluid transport, offers an attractive explanation

for the above experimental and theoretical observations, and a fascinating area for further investigation.

### 5.6 The role of intestinal $CaCO_3$ precipitation in $Ca^{2+}$ homeostasis

By drinking the surrounding seawater the rate of $Ca^{2+}$ entering the gastrointestinal tract of marine fish is essentially a continuous process and considering how tightly regulated $Ca^{2+}$ is in both the cytosol and extracellular fluids the intestine will have an important role in modulating the entry of this ion into the body. With increasing amounts of $Ca^{2+}$ entering the intestine across treatments, Figure 5.5A shows that there were increasing amounts of $Ca^{2+}$ unaccounted for in the precipitates or fluid which could amount to absorption. These potential absorption rates were 15.2 ±1.8, 37.1 ±4.1 and 80.9 ±20.4 µmol $kg^{-1}$ $h^{-1}$ from the control, 40 mM and 90 mM treatments, respectively (Figure 5.5A) and compare well with previous estimates of intestinal $Ca^{2+}$ uptake by the rainbow trout at 17.9 µmol $kg^{-1}$ $h^{-1}$ (Shehadeh and Gordon, 1969), 29.2 µmol $kg^{-1}$ $h^{-1}$ by the southern flounder, *Paralichthys lethostigma* (Hickman, 1968c) and 20.0 µmol $kg^{-1}$ $h^{-1}$ for Atlantic cod, *Gadus morhua* (Bjornsson and Nilsson, 1985).

However, additional work on $Ca^{2+}$ fluxes by the intestine of the Atlantic cod revealed that the actual rate of $Ca^{2+}$ absorption was approximately 10 times lower than these estimates (at 2.6 µmol $kg^{-1}$ $h^{-1}$), and the vast majority of this apparent absorption was in fact $Ca^{2+}$ 'trapped' within the mucus layer (Sundell and Bjornsson, 1988). A similar suggestion was made by Wilson and Grosell (2003) after finding that only 0.5-1.5 % of perfused $^{45}Ca$ could be detected in the extracellular fluid, urine and external seawater despite up to 20 % perfused $Ca^{2+}$ being unaccounted for. They considered the possibility that they had only measured $Ca^{2+}$ in precipitates that had aggregated together and separated from the mucosal surface, thus there may be a substantial portion of $Ca^{2+}$ within the mucus layer as crystalline precipitates. This is supported by the work of Humbert *et al*. (1986; 1989) finding that intestinal mucus created dense, localised concentrations of $Ca^{2+}$ from which $CaCO_3$ crystals developed in the seawater-adapted eel intestine. Since the gills do not participate in the excretion of divalent ions (Flik and Verbost, 1993) any $Ca^{2+}$ absorbed by the intestine, and not sequestered by any of the internal pools (such as bones, otoliths, scales, gonads etc.), will be removed by the kidneys which are very limited in their ability to excrete $Ca^{2+}$. Given the infrequent and small amounts of urine produced by marine

teleosts (Marshall and Grosell, 2006) it is not surprising that the renal excretion rates of $Ca^{2+}$ are low, estimated at 4.2 μmol $kg^{-1}$ $h^{-1}$ for the Atlantic cod (Bjornsson and Nilsson, 1985) and 3.3 μmol $kg^{-1}$ $h^{-1}$ for the southern flounder (Hickman, 1968c).

### 5.6.1 Calculating the rate of intestinal $Ca^{2+}$ absorption

Further evidence that this unaccounted fraction of $Ca^{2+}$ shown in Figure 5.5A was not being absorbed and sequestered by the body is illustrated by calculation of the potential $Ca^{2+}$ requirements of these fish. From the life history parameters for the European flounder presented by Saeger (1974) the average growth rate, in terms of mass, for the size of flounder used in this study was calculated to be 46.5 g per year. Unable to find any published values of whole body $Ca^{2+}$ content for the flounder, prior estimates (given per kg wet weight) for other species include 5.16 g Ca $kg^{-1}$ for rainbow trout (Shearer, 1984) and 3.35 g Ca $kg^{-1}$ for adult Atlantic salmon, *Salmo salar* (Talbot *et al*., 1986). Based on this information the $Ca^{2+}$ requirement for a year of growth was calculated as being equivalent to an intestinal absorption rate of 1.5 ±0.1 μmol $kg^{-1}$ $h^{-1}$, very similar to the prior estimate of 2.6 μmol $kg^{-1}$ $h^{-1}$ for cod using $^{45}Ca$ (Sundell and Bjornsson, 1988). Taken together, an estimate of absorption based on potential body $Ca^{2+}$ requirements along with low renal excretion rates are able to explain little more than a third of the 33 % of perfused $Ca^{2+}$ which was left unaccounted for in the control treatment.

### 5.6.2 Regulating intestinal $Ca^{2+}$ transport

Irrespective of the fate of $Ca^{2+}$ in these experiments, measurements of plasma over the course of each perfusion, revealed stable $Ca^{2+}$ concentrations across all treatments (Table 5.3) indicating no significant perturbation of whole body $Ca^{2+}$ balance which was very impressive after sustained perfusion with 90 mM $Ca^{2+}$. Similarly, Wilson and Grosell (2003) found $Ca^{2+}$ homeostasis unaffected following increases in the concentration of $Ca^{2+}$ in the surrounding seawater up to 70 mM. Even though 46 to 73 % of perfused $Ca^{2+}$ was being removed as $CaCO_3$ in the present study (Figure 5.5A) there were also substantial portions dissolved in the rectal fluid (particularly at 90 mM) suggesting the epithelium itself must also be effective at restricting excessive $Ca^{2+}$ entry into the body (Table 5.4). Therefore, discussion of the regulation of intestinal $Ca^{2+}$ absorption would not be complete

without recognising some of the endocrine factors involved in controlling and coordinating the mechanisms behind this intricate homeostasis.

Stanniocalcin (STC) and a number of vitamin D metabolites have been identified as the principal anti-hypercalcaemic factors in marine teleosts having direct effects on intestinal $Ca^{2+}$ transport. STC is produced by the corpuscles of Stannius, which are small glands located on the surface of the kidneys, and exerts its effect by decreasing the active uptake of $Ca^{2+}$ by the gills and intestine as well as the renal handling of $Ca^{2+}$ (Sundell *et al*., 1992; Flik and Verbost, 1993; Flik *et al*., 1995; Larsson, 1999). Interestingly, the secretion of STC is considered to be under the control of a CaR, where it can operate by detecting changes in circulating $Ca^{2+}$ (Radman *et al*., 2002) or *via* the caudal neurosecretory system (Ingleton *et al*., 2002). Vitamin D metabolites are lipid soluble steroid hormones, and unlike the peptide hormone STC, can cross the cell membrane thus their mechanisms of control can differ, ranging from slow genome-mediated responses such as transcriptional events of $Ca^{2+}$ binding proteins and transporters within the cell (Sundell *et al*., 1993; 1996) to rapid (within minutes) non-genome mediated effects that modulate the $Ca^{2+}$ transporting capacity of the intestine (Larsson, 1999). Interestingly, the vitamin D metabolite, 24,25-dihydroxyvitamin $D_3$ has been shown to inhibit intestinal $Ca^{2+}$ uptake in Atlantic cod (Larsson *et al*., 2002) and rainbow trout (Larsson *et al*., 2003), and the regulation of receptor expression for this and other vitamin D metabolites with similar effects on $Ca^{2+}$ transport are considered to be under the control of a calcium-sensing receptor (Larsson *et al*., 2003).

### 5.6.3 Summary

Following extended perfusion with up to 90 mM $Ca^{2+}$ there were no obvious disturbances in whole animal $Ca^{2+}$ balance. The absorption rate of $Ca^{2+}$ from the intestine is likely to be much smaller than indicated by the amounts left unaccounted for, since the portion of $Ca^{2+}$ recovered as precipitates will be greater than measured due to the time taken for aggregation of $CaCO_3$ crystals from the mucus layer. After considering the limited capacity for excretion *via* the kidneys and taking into account potential $Ca^{2+}$ requirements of these fish it can be concluded that precipitation does indeed play a key role in systemic $Ca^{2+}$ homeostasis and combined with modulation of the various $Ca^{2+}$ transport processes this presumably makes the intestine very effective at regulating $Ca^{2+}$ uptake.

# Chapter Six

## The regulation of intestinal $HCO_3^-$ secretion by the seawater-adapted killifish (*Fundulus heteroclitus* L.)

## 1. Summary

The present study set out to investigate the regulation of $HCO_3^-$ secretion by $Ca^{2+}$ using the killifish intestine, with a view to establishing a role for the calcium-sensing receptor (CaR), as well as some of the characteristics of $HCO_3^-$ secretion by this species. Experiments employed the Ussing chamber, with pH stat titration as an *in vitro* alternative to the gut sac. The baseline rates of $HCO_3^-$ secretion by the killifish intestine at 25 °C were between 0.6 and 0.8 µEq $cm^{-2}$ $h^{-1}$. Approximately 75 % of secreted $HCO_3^-$ was deemed to have originated from endogenous $CO_2$ hydration, following omission of serosal $HCO_3^-/CO_2$, and this conclusion was further supported by the insensitivity of $HCO_3^-$ secretion to serosal DIDS. Application of mucosal DIDS however, reduced $HCO_3^-$ secretion by around 37 %, offering evidence toward the presence of apical $Cl^-/HCO_3^-$ exchange, although the majority of $HCO_3^-$ secretion was likely to exit *via* an alternative conductive pathway. Despite bearing such similar characteristics to the European flounder, $HCO_3^-$ secretion by the killifish intestine did not share a similar response to mucosal $Ca^{2+}$. Increasing luminal $Ca^{2+}$ concentration from 5 to 20 mM (using $CaCl_2$) was without effect. By way of control, the addition of 15 mM $Mg^{2+}$ (as $MgCl_2$) in a separate set of experiments also left $HCO_3^-$ secretion unaffected. Similar experiments further examined the potential influence of ionic strength and/or osmolality of the mucosal saline on the response of the putative CaR to $Ca^{2+}$, but to no avail, suggesting that the stimulation of $HCO_3^-$ secretion by $Ca^{2+}$ (*via* a CaR) is not necessarily a ubiquitous trait amongst marine teleosts. The addition of both $CaCl_2$ and $MgCl_2$ also increased the concentration of mucosal $Cl^-$ by 30 mM and osmotic pressure by ~40 mOsm $l^{-1}$, resulting in a significant hyperpolarisation of the epithelia, along with an increase in ionic permeability. This was considered the result of elevated $Cl^-$ absorption from mucosa to serosa *via* a conductive pathway, such as the cystic fibrosis transmembrane regulator (CFTR). Further trials revealed a quite different osmotic stress response after increasing the osmotic pressure of the mucosal saline with the non-ionic osmolytes mannitol and sucrose, resulting in an immediate reduction in $HCO_3^-$ secretion. In some cases there was a complete abolition of luminal alkalinisation followed by gradual acidification of the mucosal saline. It was hypothesised that this response to elevated mucosal osmotic pressure was the result of a shift in the direction of $H^+$ secretion (from basolateral to apical), which would have the effect of titrating mucosal $HCO_3^-$ thus helping

reduce luminal osmotic pressure. However, subsequent experiments measuring basolateral $H^+$ secretion were unable to detect this anticipated shift in $H^+$ secretion and after ruling out a role for apical $Na^+/H^+$ exchange, further work is necessary to help understand this response.

## 2. Introduction

The failure to demonstrate a role for $Ca^{2+}$ in the stimulation of intestinal $HCO_3^-$ secretion using an *in vitro* gut sac preparation from the European flounder (Chapter 4), was very perplexing given the convincing evidence for $Ca^{2+}$-mediated $HCO_3^-$ secretion from previous *in vitro* experiments, which had employed the Ussing chamber technique with pH stat titration (Wilson *et al*., 2002). The lack of progress using the paired gut sac methodology was becoming increasingly frustrating, and continued to fuel curiosity about whether the reason for this discrepancy lay with these two *in vitro* techniques. The present chapter describes experiments conducted during a visit to the laboratory of Dr. Martin Grosell at the University of Miami (Florida, USA), which was an exciting opportunity to gain some experience of the Ussing chamber technique (with pH stat) and apply it as part of investigations into the regulation of intestinal $HCO_3^-$ secretion by $Ca^{2+}$. It was hoped that this would result in progress toward demonstrating a potential, functional role for a calcium-sensing receptor (CaR) in the marine teleost intestine.

### 2.1 The Ussing chamber

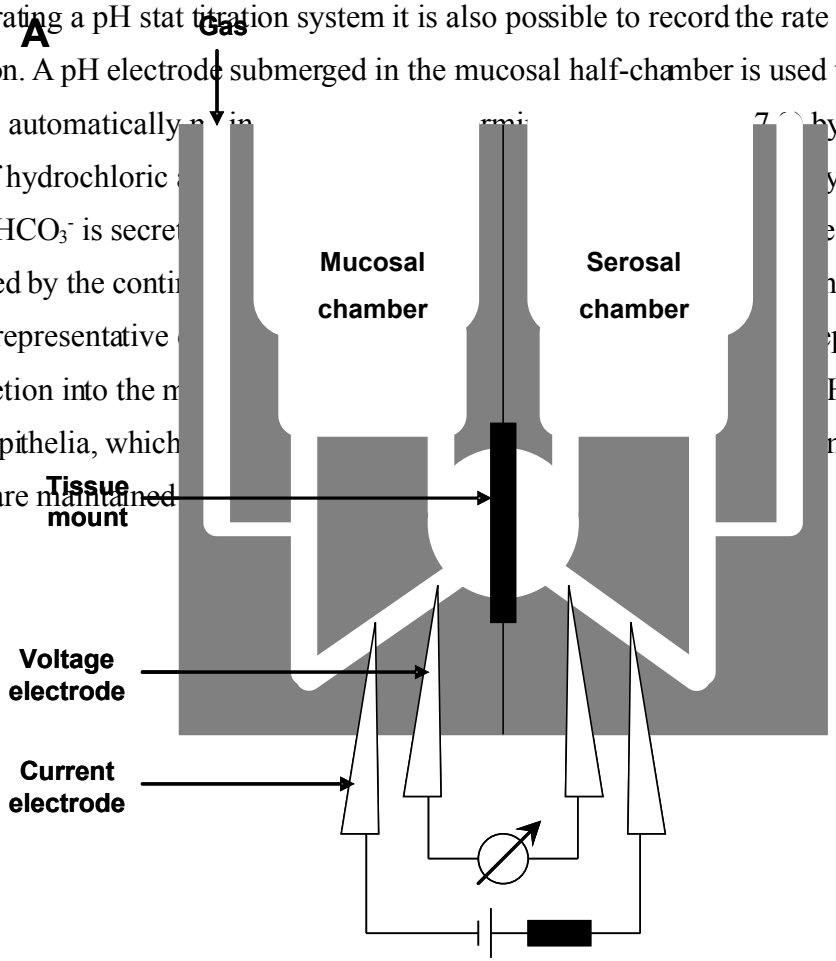
Named after its inventor, the late Danish physiologist Hans Ussing, the Ussing chamber was introduced in the mid-twentieth century as a means of studying ion transport and equivalent electric currents across isolated frog skin (Ussing and Zerahn, 1951). Interestingly, this work is credited, amongst others, with helping set the stage for the modern era of epithelial transport physiology (Schultz, 1998). Furthermore, from what was a relatively simple design concept this technique has become a very powerful tool for studying the properties of transporting epithelia *in vitro*, finding a broad range of applications in cell and tissue research.

Compared with the gut sac preparation described in previous chapters, the Ussing chamber set up consists of the isolated, excised epithelium mounted as a flat sheet, typically in a vertical orientation, secured between two half-chambers which connect together to form a single unit (chamber) with the mucosal and serosal sides separated by a small, exposed area of tissue (Figure 6.1). Unlike the mucosal saline which is sealed within the gut sac and not gassed or stirred, leading to potentially limiting effects on transport processes (discussed in Chapter 4, Section 5.1), these artifacts are minimised in the Ussing chamber as both sides of the tissue are simultaneously gassed and stirred thus providing the assurance of adequate oxygenation as well as reducing unstirred layer effects.

With such a small area of epithelia exposed to a considerably larger volume of saline the ratio of chamber volume to surface area makes it extremely difficult to monitor fluid movements by the tissue (likely to be in the nano-litre range). However, with gut sacs this ratio is reversed permitting the recording of measurable volume flows across the epithelia along with simultaneous measurements of net solute transport. In contrast, the transport of ions by epithelia in the Ussing chamber is typically represented as equivalent electrical currents and/or fluxes of specific radioactive tracers.

### 2.2 Measuring $HCO_3^-$ secretion in the Ussing chamber

By incorporating a pH stat titration system it is also possible to record the rate of mucosal alkalinisation. A pH electrode submerged in the mucosal half-chamber is used to monitor pH which is automatically m in rmi 7.0 by small additions of hydrochloric a y micro-burette. As $HCO_3^-$ is secret e resulting $CO_2$ removed by the conti n is therefore considered representative eparation, $HCO_3^-$ secretion into the m H gradient across the epithelia, which mucosal and serosal pH are maintained



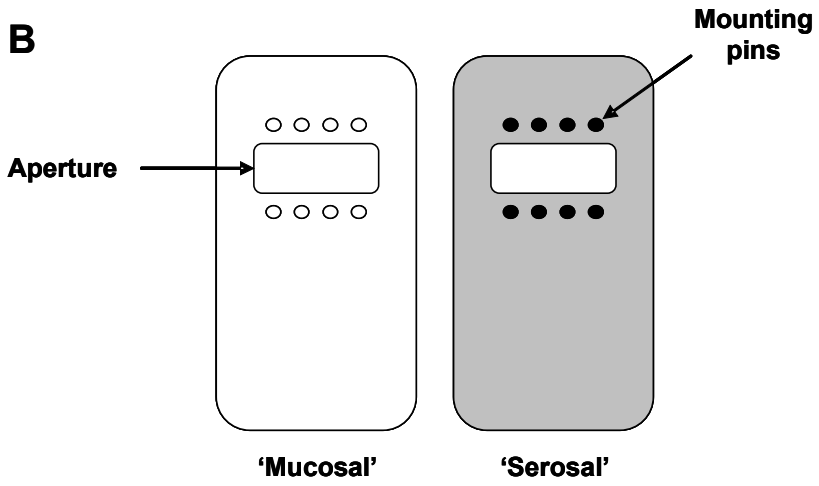



Figure 6.1: A) A schematic drawing of a circulating Ussing chamber based on the design used in the present study. B) A plan view of the two halves of the tissue mount inserted between the two half chambers. A small flat section of intestine with the mucosal surface uppermost was placed across the aperture of the serosal mount, held in place by pins on either side. The mucosal half of the mount was then placed on top, leaving the mucosal and serosal surfaces of the tissue exposed, ready to be secured between the two halves of the Ussing chamber as shown in part A.

In previous studies, the Ussing chamber technique, with pH stat titration, has been successfully applied to investigations of intestinal $HCO_3^-$ secretion by a number of marine species including the Japanese eel, *Anguilla japonica* (Ando and Subramanyam, 1980), goby, *Gillichthys mirabilis* (Dixon and Loretz, 1986), European flounder (Wilson *et al*., 2002; Wilson and Grosell, 2003) and toadfish, *Opsanus beta* (Grosell and Genz, 2006).

**2.3 The euryhaline killifish**

The species chosen for this work was the common killifish or mummichog (*Fundulus heteroclitus*) which is found along the eastern coast of North America from Texas on the Gulf of Mexico to the Gulf of St. Lawrence, Canada (Scott and Scott, 1988). The killifish is a small, inter-tidal species occupying salt marshes and estuaries in coastal regions where it is likely to encounter regular fluctuations in salinity, as well as other potential environmental variables such as dissolved oxygen, pH and temperature (Burnett *et al*., 2007). The physiological adaptations and tolerances of this species together with its abundance, ease of collection and amenity to aquarium conditions, have made the killifish a popular experimental model (Atz, 1986; Burnett *et al*., 2007).

The killifish is perhaps most renowned for its broad tolerance of salinity, ranging from freshwater through to hyper-salinities of up to 120 ppt, 4 times stronger than regular seawater (Griffith, 1974). In common with other teleosts, the killifish drinks at comparable rates when in hyper-osmotic media (Potts and Evans, 1967; Malvin *et al*., 1980; Scott *et al*., 2006). The gastrointestinal tract is extremely simple and does not possess a stomach instead the oesophagus joins directly to the anterior intestine, the latter acting as a receptacle for food (Babkin and Bowie, 1928). To date, the majority of investigations into the euryhalinity of this species have largely focussed on the function of the gills, in particular the chloride cells of the opercular epithelium, (reviewed by Karnaky, 1986; Wood and Marshall, 1994; Hoffman *et al*., 2007). The ion transporting characteristics of the intestine appear to have received considerably less attention, being represented by only a handful of studies, including Marshall *et al*. (2002) and Scott *et al*. (2006). In spite of this, the intestine of the killifish does display substantial rates of intestinal $HCO_3^-$ secretion (M. Grosell, personal communication), and with intestinal $Cl^-$ fluxes in excess of $Na^+$ (Scott *et al*., 2006), suggests a role for $Cl^-/HCO_3^-$ exchange (Grosell, 2006).

The killifish was therefore considered a suitable model to continue exploring the mechanisms behind $HCO_3^-$ secretion in seawater-adapted teleosts. It would be very interesting to discover whether the stimulation of intestinal $HCO_3^-$ secretion by $Ca^{2+}$ is a ubiquitous trait amongst teleost species. For example, olfactory sensitivity to changes in environmental $Ca^{2+}$ concentrations is considered a widespread phenomenon in teleosts having been demonstrated for euryhaline (Bodznick, 1978; Hubbard *et al*., 2000) and freshwater species (Hubbard *et al*., 2002), along with compelling evidence that these responses are conferred by a calcium-sensing receptor, CaR (Hubbard *et al*., 2002). As yet the CaR from the killifish has not been fully described, and appears limited to a brief report which found significantly increased mRNA expression for a CaR in the gills and kidney following transfer from freshwater to seawater (Baum *et al*., 1996).

**2.4 Does ionic strength influence intestinal $HCO_3^-$ secretion?**

The (human parathyroid) CaR is known to be modulated by the ionic strength (but not osmolality) of the surrounding medium, whereby sensitivity of the receptor to its agonists is enhanced following a reduction in ionic strength (Quinn *et al*., 1998). Based on these findings it was subsequently proposed that the prevalence of monovalent ions, such as $Na^+$, at higher ionic strengths will act to shield polycationic ligands (i.e. $Ca^{2+}$ and spermine) from attaching to their binding sites on the extracellular domain of the CaR, hence reducing their affinity for the receptor (Quinn *et al*., 1998). These observations on the mechanism of receptor function are also shared by CaRs from fish, including the dogfish (*Squalus acanthias*), where activation of the CaR by either $Ca^{2+}$ or $Mg^{2+}$ is indirectly proportional to NaCl concentrations (Nearing *et al*., 2002; Fellner and Parker, 2004). Similar $Ca^{2+}$-sensing dependence on ionic strength has also been confirmed for the tilapia, *Oreochromis mossambicus* (Loretz *et al*., 2004). Together these observations support the conclusion that the CaR in teleosts acts as a salinity sensor (Nearing *et al*., 2002), and the receptor is thought to have originally evolved as part of the osmoregulatory strategy in fish (Hebert, 2004; Loretz, 2008).

Assuming that a CaR is indeed involved in the regulation of intestinal $HCO_3^-$ secretion as suggested by Wilson *et al*. (2002), this raised the question of whether ionic strength may influence CaR function. For example, as seawater is processed along the intestine the concentration of $Na^+$ is typically reduced from around 170 mM as it enters the anterior

intestine from the stomach, to 30 mM in the rectum (Personal observations from the European flounder). Based on the changes in $EC_{50}$ for receptor activation versus extracellular $Na^+$ concentration for the CaR isolated from the dogfish kidney (Nearing *et al*., 2002), and assuming a similar range of sensitivity for an intestinal CaR, this change in $Na^+$ concentration along the gut would reduce the $EC_{50}$ of the CaR for $Ca^{2+}$ from around 9 to 2 mM. Thus, as seawater is processed along the intestine the sensitivity of the CaR to $Ca^{2+}$ could be enhanced more than 4-fold. If $HCO_3^-$ secretion by the killifish intestine were found to be stimulated by $Ca^{2+}$ then manipulating the ionic strength of the mucosal saline may offer some valuable insight into the underlying mechanism and the regulation of intestinal $HCO_3^-$ secretion *in vivo*. Alternately, if $Ca^{2+}$ failed to stimulate $HCO_3^-$ secretion then these experiments would be equally useful to rule out the composition of the mucosal saline as a factor.

### 2.5 Aims and objectives

Employing the Ussing chamber in conjunction with a pH stat system, the aim of this study was to determine whether elevated luminal $Ca^{2+}$ concentration stimulated $HCO_3^-$ secretion by the intestine of the euryhaline killifish. Experiments also sought to address the role of ionic strength and osmotic pressure in further modulating intestinal $HCO_3^-$ secretion.

## 3. Materials and Methods

### 3.1 Experimental animals

Killifish, *Fundulus heteroclitus* (n = 63, body mass 3.90 ±0.12 g and 6.7±0.1 cm total length) from St. Augustine (FL, USA) and Woods Hole (MA, USA) were shipped to the aquarium facilities at the University of Miami, Rosenstiel School of Marine and Atmospheric Sciences. On arrival the fish were quarantined and subject to a prophylactic treatment for ectoparasites consisting of malachite green (final concentration 0.05 mg $l^{-1}$) in formalin (15 mg $l^{-1}$) (Aquavet, CA, USA), and afterwards held in 80 litre glass tanks receiving a continuous flow of filtered, aerated seawater (salinity 33-35 ppt, 22-26 °C from Bear Cut, FL). Food was typically withheld for 24-48 hours prior to experimentation

otherwise fish were fed a commercial pellet feed (Aquatic Eco-Systems, FL, USA) to satiation every other day.

### 3.2 General experimental protocol

Fish were terminated by a sharp blow to the head, destroying the brain. An incision was made just behind the pelvic girdle and the abdominal cavity opened by carefully cutting along the ventral axis of the body towards the head until level with the pectoral fins. The intestine was gently exposed and a section (approximately 1 cm in length) was cut from the anterior region and opened up to a flat sheet with a single longitudinal cut and carefully mounted in the tissue mount exposing a gross surface area of 0.3 $cm^2$. The tissue was subsequently secured between two half chambers (P2400, Physiologic Instruments, CA, USA) and bathed with 1.6 ml of the appropriate, pre-gassed saline in each half-chamber. Each saline was simultaneously mixed and gassed through airlifts with an appropriate humidified gas mixture, typically 100 % $O_2$ in the mucosal saline and 0.3 % $CO_2$ ($O_2$ balance) in the serosal saline (Table 6.1), to mimic *in vivo* serosal conditions. The Ussing chamber was mounted in a thermostatically controlled chamber holder and the preparation maintained at 25 °C.

Current and voltage electrodes were connected to an amplifier (VCC600, Physiologic Instruments) recording the transepithelial potential (TEP), with the current clamped at 0 µA. TEP was measured in mV in relation to the serosal saline, and was always negative indicating an overall, net active transport of negative charge from mucosa to serosa. To measure transepithelial conductance ($G_t$), the amplifier was set up to deliver a current pulse of 30 µA (of 3 second duration) every 60 seconds across the tissue, from mucosa to serosa, and based on the resulting change in TEP allowed for calculation of $G_t$ using Ohms Law (equation 1).

To permit current pulsing during pH stat titration the auto-titrator was grounded to the amplifier. Each chamber was supplied with 4 electrodes (2 × Ag/AgCl pellet electrodes for voltage and 2 × Ag wire electrodes for current) which were connected to the mucosaland serosal salines by agar-salt bridges. To create the agar bridge tapered polyethylene electrode tips (Physiologic Instruments) were part-filled with a heated solution of 2 % agar (w/v) dissolved in 3 M KCl and allowed to set. The electrodes were then screwed into these polyethylene tips which had been filled with 3 M KCl, on top of the set agar (while

avoiding any air bubbles), and the agar bridges fitted into the front of chamber (Plate 1). Both current and voltage measurements could then be logged onto a personal computer using BIOPAC Systems (CA, USA) interface hardware and AcqKnowledge software (version 3.8.1).

The pH-stat titrations were performed on the mucosal saline (except during measurement of basolateral proton secretion, see further on), by placing a combination pH electrode (PHC4000.8, Radiometer, Denmark), and tip of a micro-burette (ABU901, Radiometer) into the mucosal half of the chamber, which were connected to an auto-titration system (TIM 854 or 856, Radiometer). The completed experimental set up, with all aspects of the Ussing chamber plus pH stat titration in place, is shown in Plate 6.1.

Once the tissue had stabilised and secreted sufficient $HCO_3^-$ equivalents ($HCO_3^- + 2CO_3^{2-}$) for the mucosal saline pH to reach 7.8 (typically, 30 to 90 minutes), the pH stat titration automatically commenced. Mucosal pH was maintained at 7.800 (±0.003 pH units) by the addition of acid (0.005 N HCl) delivered *via* micro-burette which titrated the $HCO_3^-$ equivalents to volatile $CO_2$, which was removed by gassing with 100 % $O_2$. The rate of acid addition and mucosal saline pH were logged onto a personal computer using Titramaster software (versions 1.3 and 2.1) every 10 seconds. The secretion rates of $HCO_3^-$ equivalents were calculated from linear regression analysis of the amount of acid required to maintain pH at 7.8 over each 10 minute time period and were reported as a function of surface area (cm
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rate
var

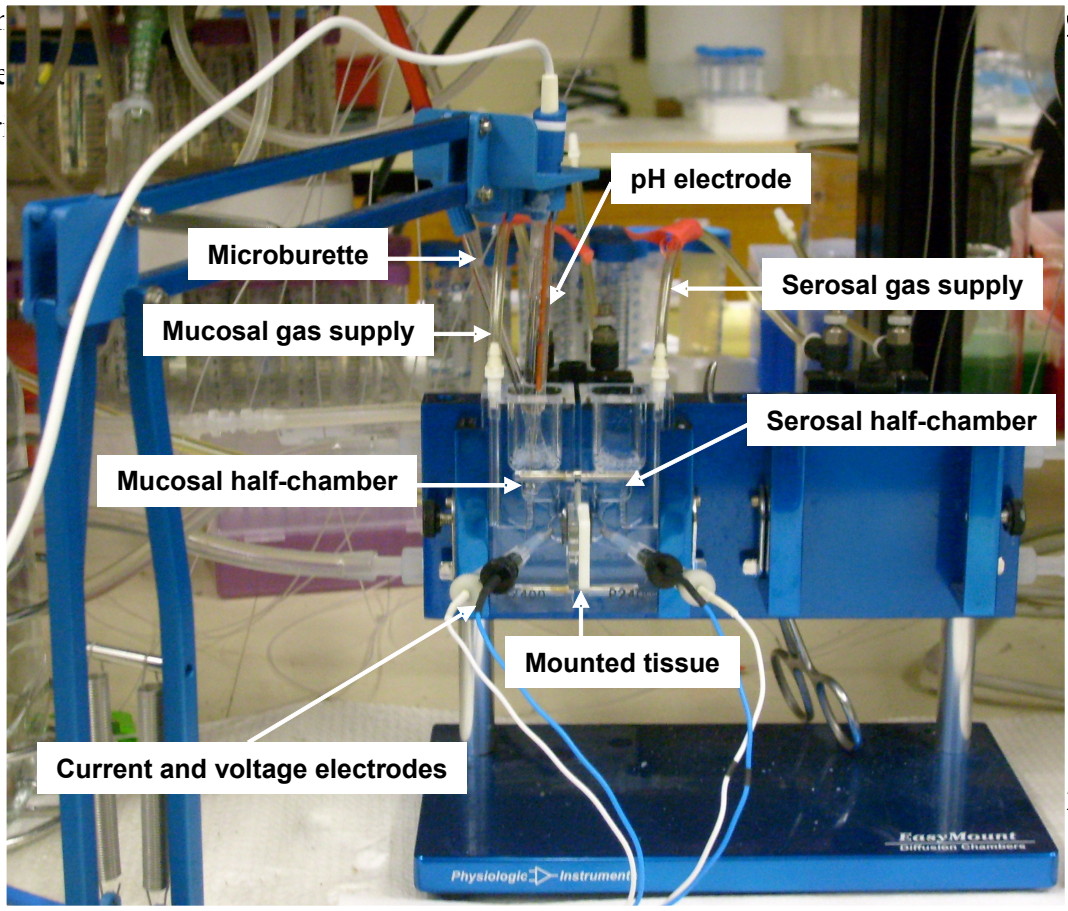


Plate 6.1: The Ussing chamber set up with pH stat (micro-burette and pH electrode) showing the tissue mounted between the two half chambers. Separate gas supply lines provided simultaneous gassing and mixing of the mucosal and serosal salines bathing the tissue in each half chamber. A set of current-passing electrodes (white) and voltage-sensing electrodes (black) are fitted into each half chamber *via* an agar-salt bridge to monitor the electrophysiological parameters of the tissue. The wires leading from the electrodes on the serosal side are grounded to the auto-titrator to enable current pulsing during the recording of pH stat titrations.

### 3.3 Stimulation of $HCO_3^-$ secretion by $Ca^{2+}$

Using the regular mucosal and serosal salines listed in Table 6.1 the first set of experiments set out to investigate whether $HCO_3^-$ secretion by the killifish intestine is stimulated by $Ca^{2+}$, as observed for the European flounder using the same *in vitro* experimental approach described by Wilson *et al*. (2002). Once the control period was established, the mucosal saline was spiked with 15 mM $Ca^{2+}$ (added as 1 M $CaCl_2$), thus raising the $Ca^{2+}$ concentration from 5 mM to 20 mM, and the effects on $HCO_3^-$ secretion and electrophysiological parameters were followed for a further 60 minutes. To confirm that any response was specific to $Ca^{2+}$ and not a result of the increase in osmotic pressure or $Cl^-$ concentration, a separate set of experiments were carried out but spiking the mucosal saline with 15 mM $Mg^{2+}$ (added as 1 M $MgCl_2$) instead.

### 3.4 Is $HCO_3^-$ secretion modulated by ionic strength and/or osmolality?

The calcium-sensing receptor (CaR) is considered to act as a salinity sensor in marine teleosts (Nearing *et al*., 2002) and the sensitivity of the receptor to its primary ligand (i.e. $Ca^{2+}$) is modulated by the ionic strength (but not osmolality) of the surrounding medium

(Quinn *et al*., 1998; Nearing *et al*., 2002; Fellner and Parker, 2004; Loretz *et al*., 2004). Assuming the involvement of a CaR in the stimulation of $HCO_3^-$ secretion by the killifish intestine the next set of experiments set out to test whether the response of $HCO_3^-$ secretion to $Ca^{2+}$ is similarly attenuated by altering the osmotic pressure or ionic strength of the bathing salines. The first of these trials employed a mucosal saline of reduced ionic strength (but with the osmotic pressure compensated by mannitol, see Table 6.1) and the mucosal half-chamber spiked with 15 mM $Ca^{2+}$, following a control period and monitoring the effects on the tissue. As before, these same experiments were repeated but with the addition of 15 mM $Mg^{2+}$ in place of $Ca^{2+}$. The next set of trials employed the same approach (spiking the mucosal half-chamber with $Ca^{2+}$ and $Mg^{2+}$), but used a reduced osmolality mucosal saline (i.e. without mannitol). These experiments also required the design of a serosal saline with reduced osmolality to ensure the absence of any transepithelial osmotic gradient, while maintaining an ionic composition similar to what would be experienced by the tissue *in vivo* (Table 6.1).

### 3.4.1 Effects of mucosal hyperosmolarity

The additions of $CaCl_2$ and $MgCl_2$ will not only increase ionic strength (by 45 mM) but also the osmotic pressure of the mucosal saline by around 40 mOsm $kg^{-1}$. In addition to the possibility of $Ca^{2+}$-mediated regulation of $HCO_3^-$ secretion, Grosell *et al*. (2007) have predicted that luminal osmotic pressure may trigger a shift in the direction of $H^+$ secretion from basolateral to apical. As discussed in the previous chapter (Chapter 4, Section 5.2.2), this would effectively titrate secreted $HCO_3^-$ (to $CO_2$ and $H_2O$) and consequently benefit fluid absorption by reducing the osmotic pressure within the lumen. The following experiments therefore assessed the influence of raising osmotic pressure only, by initially spiking the mucosal saline with an impermeable solute, either mannitol or sucrose (added from a concentrated 1 M stock solution), to increase osmolality by approximately 40 mOsm $kg^{-1}$. After an hour the osmotic pressure of the serosal saline was subsequently increased, restoring the transepithelial osmotic gradient and measurements recorded for a further 60 minutes. For these, and the remainder of experiments detailed below, the reduced osmolality mucosal and serosal salines were employed (Table 6.1).

### 3.4.2 Source of $HCO_3^-$ secretion

Considering the predicted effects of mucosal hyperosmolarity leading to a change in the polarity of $H^+$ secretion, it was necessary to assume that a large portion of secreted $HCO_3^-$ must arise from intracellular $CO_2$ hydration, similar to other species such as the flounder (Wilson and Grosell, 2003) and toadfish (Grosell and Genz, 2006). However, preliminary experiments have suggested that the killifish intestine is in fact largely reliant on serosal $HCO_3^-/CO_2$ (Janet Genz, personal communication). To investigate this further the serosal saline was substituted for a $HCO_3^-$-free, buffered saline (Table 6.1). Once the control period had passed the regular serosal saline was removed, the half-chamber rinsed twice, before refilling with 1.6 ml of $HCO_3^-$-free, serosal saline which was then gassed with 100 % $O_2$.

### 3.4.3 Basolateral $H^+$ secretion in relation to mucosal osmotic pressure

If intracellular $CO_2$ hydration was shown to be a significant source for the supply of $HCO_3^-$ for apical secretion then substantial basolateral $H^+$ secretion would be necessary to sustain these processes (Grosell *et al.*, 2005; Grosell, 2006; Grosell and Genz, 2006). This would also offer the opportunity to detect any change in the direction of $H^+$ secretion (from basolateral to apical) following an increase in mucosal osmolarity. The pH stat system was subsequently modified to measure the rate of serosal acidification. The titrant was changed to 0.005 N NaOH, rather than HCl, with the pH set point remaining at 7.800, typical of teleost blood pH. The pH electrode and micro-burette were then set up in the serosal half-chamber where the serosal saline was completely unbuffered, containing neither HEPES, $HCO_3^-$ nor $PO_4^{2-}$ and gassed with 100 % $O_2$ (Table 6.1). Experiments were conducted in the same manner as described for the measurement of mucosal $HCO_3^-$ secretion (Section 3.2).

### 3.4.4 Mediation of apical $HCO_3^-$ and $H^+$ secretion

With the time constraints of this particular study, the final series of experiments attempted to identify the transporters behind $HCO_3^-$ secretion and putative apical $H^+$ secretion. While substantial physiological data has accumulated in support of the involvement of $Cl^-/HCO_3^-$ exchange on the apical, and in some cases the basolateral membrane for the majority of species examined (reviewed by Grosell, 2006), this has still to be fully characterised for the killifish. Subsequently, experiments were carried out applying the well known anion transport inhibitor DIDS (4,4'-di-isothiocyanatostilbene-2,2'-disulfonic acid disodium salt). DIDS was dissolved in DMSO and after the 60 minute control period was applied to the

mucosal half-chamber giving a final concentration of $10^{-4}$ M DIDS in 0.1 % DMSO, and measurements followed for a further 60 minutes before application to the serosal saline. The $Na^+$ channel blocker amiloride was also used in a separate set of experiments to assess the presence of an apical $Na^+/H^+$ antiporter. These were performed by a mucosal application of amiloride, again dissolved in DMSO, and presented at a final concentration of $10^{-4}$ M in 0.1 % DMSO.

### 3.5 Buffer capacity of mannitol and sucrose

To rule out the possibility that the addition of osmolytes, mannitol and sucrose offered any significant buffering capacity to the mucosal saline and could therefore influence the alkalinisation rate of the mucosal saline a separate set of titrations were carried out on arriving back at the University of Exeter. The auto-titrator employed was the same model used for the previous pH-stat experiments (TIM 845 titration manager, Radiometer) but with a different combination pH electrode (pHC2401-8, Radiometer) and burette (ABU52, Radiometer). Before commencing titration, a 20 ml aliquot of saline was pre-gassed for 1 hour with 100 % $N_2$ to remove $CO_2$ (similar to the pre-gassing routine during actual pH-stat experiments which used 100 % $O_2$). After this initial equilibration the pH of the saline was recorded and 0.0005 N NaOH added steadily at a rate of 0.30 ml $min^{-1}$ while the sample was continuously gassed and stirred, and the rise in pH monitored. All pH readings (to within ±0.003 pH units) and volume of base added (to the nearest µl) were logged onto a personal computer using Titramaster 85 software (version 3.1). The starting pH of all the salines was ~6.5, thus the buffer capacity of each saline was subsequently calculated as the amount of base required to raise the saline pH from 7.000 to 7.800 remaining relevant to the pH stat experiments. Buffer capacity was measured for the reduced osmolality saline (Table 6.1), and subsequently with salines containing either 40 mM mannitol or sucrose.

### 3.6 Saline design and composition

The salines used in these experiments are presented in Table 6.1 and based on the ionic composition of the intestinal fluid and blood plasma from the killifish. On the morning of each experiment, glucose was added to the serosal saline (and mannitol to the mucosal saline as necessary) before measuring osmolality (Wescor Vapro 5520) and adjusting as required with deionised water so that both salines yielded an identical osmotic pressure.

Each saline was then gassed continuously for at least 60 minutes with the appropriate gas mixture prior to being aliquoted into the Ussing chamber.

### 3.7 Calculations

The resultant changes to TEP (ΔPD) and current (ΔI) from baseline values during pulsing were calculated for every 5 minute time period over the course of each experiment and used to derive the transepithelial conductance ($G_t$, presented as mS $cm^{-2}$) at each time point according to Ohms Law and calculated as:

$$G_t = \Delta I / (\Delta PD \times 0.3) \qquad (1)$$

where, 0.3 is the gross surface area ($cm^2$) of the exposed epithelium.

Table 6.1: The composition of the mucosal and serosal salines employed in the following experiments displaying the concentration of each component (given in mmol $l^{-1}$), along with the measured pH of the serosal saline (following equilibration with respective gas mixture). Osmolarity was calculated from the given osmotic coefficient (Robinson and Stokes, 1965) of each component and presented as mOsm $l^{-1}$.

| | **Mucosal salines** | | | **Serosal salines** | | | |
|---|---|---|---|---|---|---|---|
| | **Regular** | **Reduced ionic strength** | **Reduced osmolality** | **Regular** | **Reduced osmolality** | **$HCO_3^-$ free** | **Buffer free** |
| NaCl | 90.0 | 45.0 | 45.0 | 146.0 | 132.0 | 132.0 | 141.0 |
| KCl | 5.0 | 5.0 | 5.0 | 3.0 | 2.0 | 2.0 | 3.0 |
| $MgSO_4.7H_2O$ | 80.0 | 80.0 | 80.0 | 0.9 | 0.9 | 0.9 | 0.9 |
| $MgCl_2.6H_2O$ | 15.0 | 15.0 | 32.5 | - | - | - | - |
| $CaCl_2.6H_2O$ | 5.0 | 5.0 | 5.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| $NaHCO_3$ | - | - | - | 8.0 | 8.0 | - | - |
| $Na_2HPO_4$ | - | - | - | 0.5 | 0.5 | 0.5 | - |
| $K_2HPO_4$ | - | - | - | 0.5 | 0.5 | 0.5 | - |
| HEPES (Free acid) | - | - | - | 4.0 | 4.0 | 4.0 | - |
| HEPES ($Na^+$ salt) | - | - | - | 4.0 | 4.0 | 4.0 | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Glucose | - | - | - | 6.0 | 6.0 | 6.0 | 6.0 |
| Mannitol | - | 80.0 | - | - | - | - | - |
| Osmolarity | 319 | 319 | 282 | 314 | 286 | 270 | 286 |
| pH | 7.80* | 7.80* | 7.80* | 7.83 | 7.83 | 7.80 | 7.80* |
| Gas mixture | | 100 % $O_2$ | | 0.3 % $CO_2$ ($O_2$ balance) | | 100 % $O_2$ | |

*pH set point of the pH stat titration

### 3.8 Data presentation and statistics

The data are presented as means (±SE) and sequential paired t-tests used to evaluate differences between individual time points post-treatment with a control value. The control value was calculated from the average of the last 30 minutes of the control period prior to treatment. To reduce the type-1 error rate following multiple paired comparisons a modified Bonferroni correction of the probability values associated with each test was applied (Holm, 1979; Sokal and Rohlf, 1994). Significant differences in buffer capacity between mucosal salines were assessed by the non-parametric Kruskal-Wallis test, as the data failed to meet the assumptions of normality and equality of variance associated with a one-way ANOVA, and post-hoc, pair-wise comparisons were made using Dunns procedure. The results of all tests were accepted as significant at $P < 0.05$. Statistical analyses were carried out using Minitab v13.1 and graphs drawn with SigmaPlot v9.0.

## 4. Results

### 4.1 Stimulation of $HCO_3^-$ secretion by $Ca^{2+}$

There was no effect of increasing mucosal $Ca^{2+}$ on $HCO_3^-$ secretion by the killifish intestine using the Ussing chamber and pH stat system (Figure 6.2C). Electrophysiological measurements showed that addition of either $CaCl_2$ or $MgCl_2$ led to a rapid and significant hyper-polarisation of the epithelia (Figures 6.2A and D), combined with a significant increase in conductance (Figure 6.2B and C), suggesting an increase in net, overall negative ionic flux and the permeability of the epithelia.

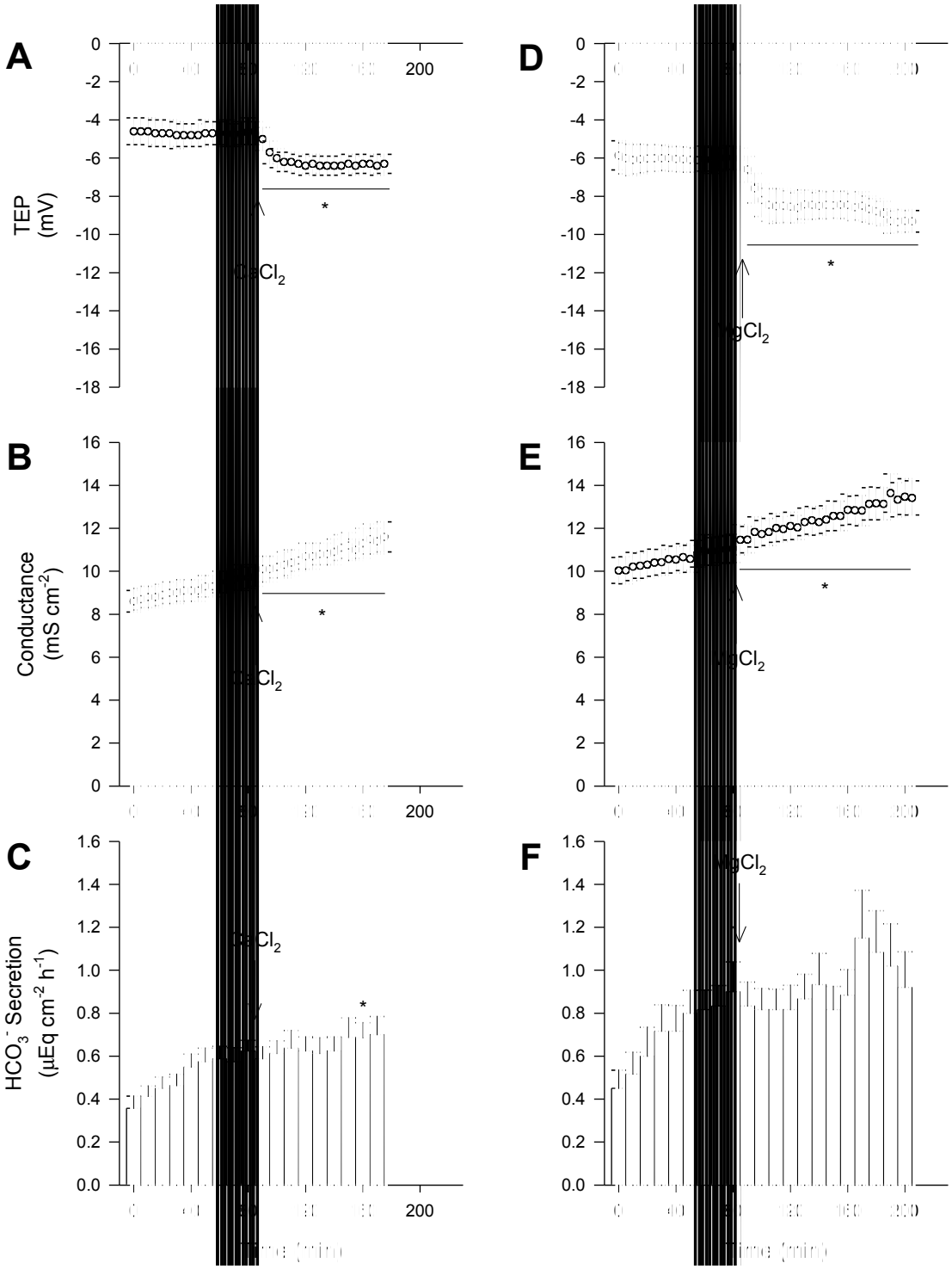



Figure 6.2: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^{-2}$ $h^{-1}$) from the isolated anterior intestine of the killifish following the addition of 15 mM $CaCl_2$ (Panels A to C), or 15 mM $MgCl_2$ (Panels D to F) to the mucosal saline. Results are means ±SE based

on n = 8 and 6 for the $CaCl_2$ and $MgCl_2$ treatments, respectively. Means with asterisks are significantly different from the preceding 30 minute control period, indicated by filled circles or bars ($P < 0.05$).

**4.2 Is $HCO_3^-$ secretion modulated by ionic strength and osmolality?**

Once again in the presence of 20 mM $Ca^{2+}$ there was no stimulation of $HCO_3^-$ secretion, in fact there was an average 28 % decrease, although this was found to not be statistically significant (Figure 6.3C). The corresponding set of 'control' experiments with $MgCl_2$ were overshadowed by problems and preparations would struggle to reach pH 7.8 and even begin the pH stat titration. For those tissue preparations that were successful, baseline rates of $HCO_3^-$ secretion were noticeably depressed, by approximately 50 % (Figure 6.3F), compared with previous experiments using $CaCl_2$. However, in spite of the reduced rate of $HCO_3^-$ secretion TEP appeared unaffected (Figures 6.3A and D), as well as conductance (Figures 6.3B and E), and in general the baseline electrophysiological parameters were comparable between treatments and demonstrated the same response to both $CaCl_2$ and $MgCl_2$.

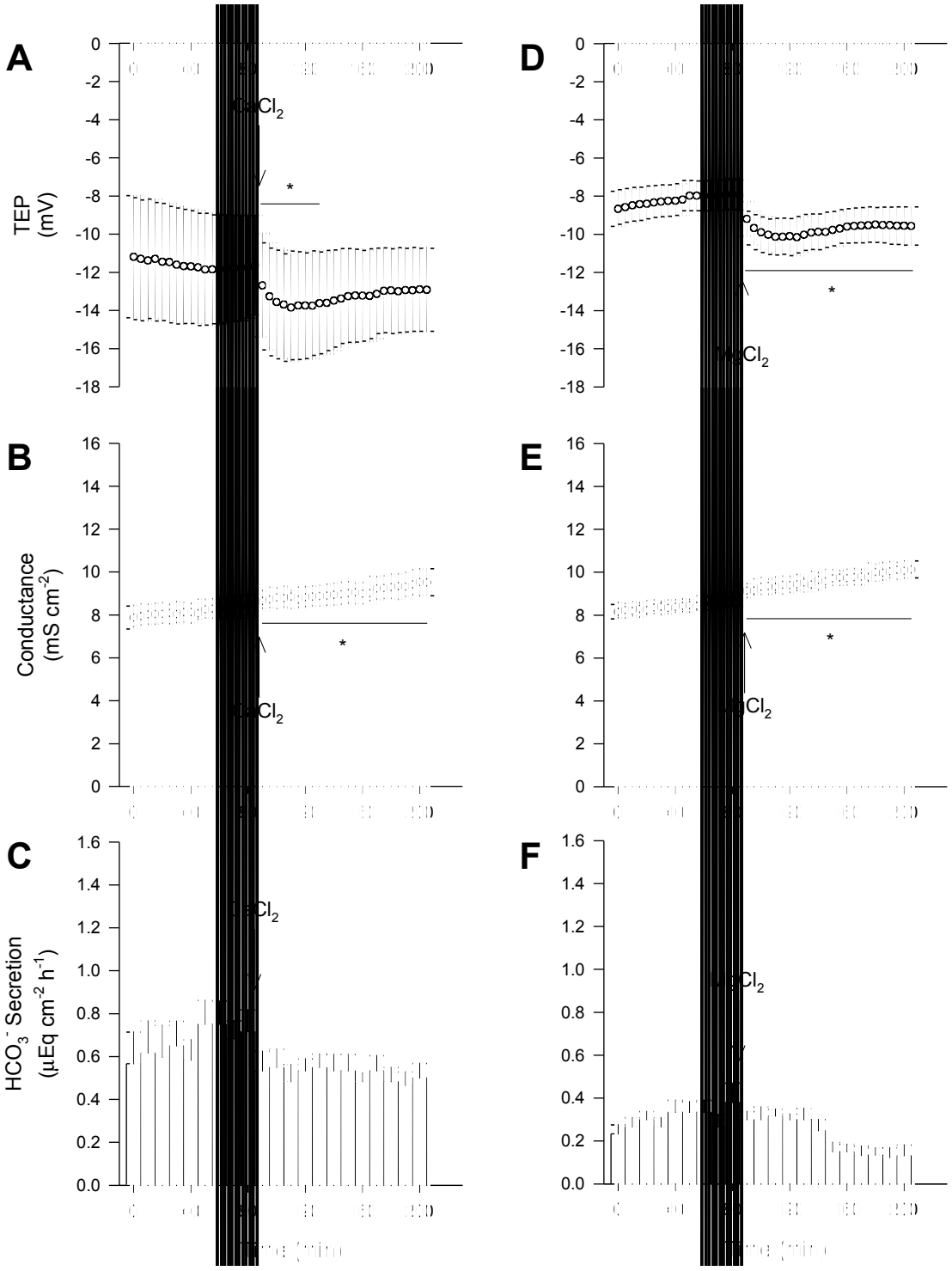



Figure 6.3: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^{-2}$ $h^{-1}$) from the isolated anterior intestine of the killifish following the addition of 15 mM $CaCl_2$ (Panels A to C), or 15 mM $MgCl_2$ (Panels D to F) to the 'reduced ionic strength' mucosal saline.

Results are means ±SE based on n = 6 for both $CaCl_2$ and $MgCl_2$ treatments. Means with asterisks are significantly different from the preceding 30 minute control period, indicated by filled circles or bars ($P < 0.05$).

The third manipulation of the mucosal saline composition in an attempt to resolve the potential for $Ca^{2+}$ to stimulate $HCO_3^-$ secretion involved reducing the osmotic pressure. Interestingly, after switching from the previous 'reduced ionic strength' saline the problem described for the previous $MgCl_2$ treatment (Figure 6.3F) had disappeared and Figures 6.4C and F show that control $HCO_3^-$ secretion was restored to baseline rates (between 0.6 and 0.8 $\mu Eq\ cm^{-2}\ h^{-1}$). The addition of $CaCl_2$ or $MgCl_2$ did not influence secretion rates, and was accompanied by a significant increase in conductance (Figures 6.4B and E) but a smaller reduction in TEP than seen previously (Figures 6.4A and D).

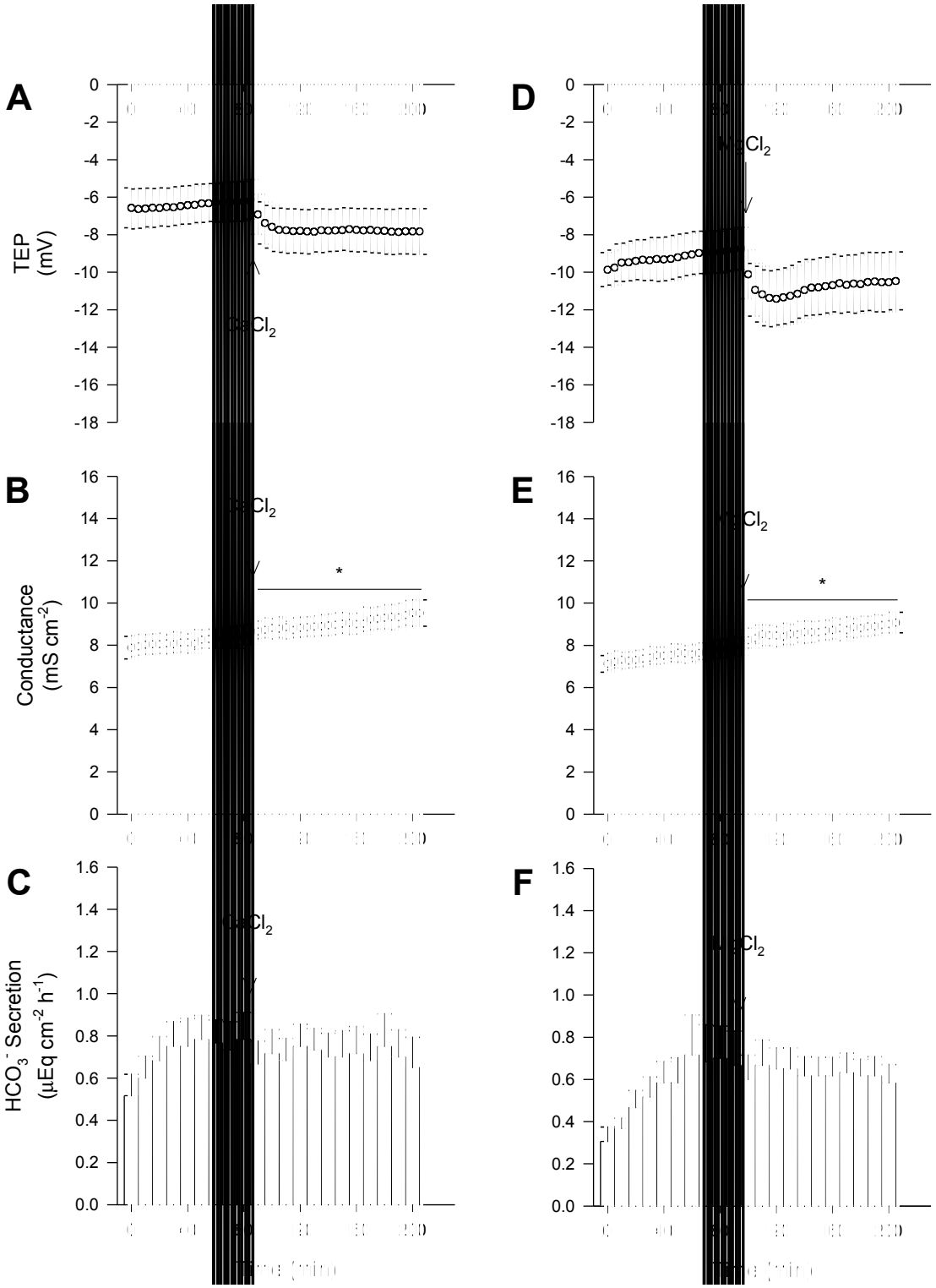



Figure 6.4: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^{-2}$ $h^{-1}$) from the killifish intestine following the addition of 15 mM $CaCl_2$ (Panels A to C), or 15 mM $MgCl_2$ (Panels D to F) to the 'reduced osmolality' mucosal saline. Results are means ±SE based on

n = 6 for both $CaCl_2$ and $MgCl_2$ treatments. Means with asterisks are significantly different from the preceding 30 minute contrd period, indicated by filled circles or bars ($P < 0.05$).

### 4.2.1 Effects of mucosal hyperosmolarity

In response to an increase in osmotic pressure only (in the absence of any change in ionic strength) by the application of mannitol to the mucosal saline resulted in a rapid 40 % reduction in $HCO_3^-$ secretion, but was unfortunately not statistically significant (Figure 6.5C). There were no significant effects on electrophysiological parameters, but in contrast to previous experiments there appeared to be a gradual reduction in TEP (ionic flux) and an increase in conductance (permeability) (Figures 6.5A and B). A corresponding application of mannitol to the serosal side, thus effectively removing the imposed transepithelial osmotic gradient, did not restore $HCO_3^-$ secretion (Figure 6.5C). To help confirm that this was a result of osmotic pressure and not a specific response to mannitd, these experiments were repeated using sucrose as a non-absorbable osmolyte. Reassuringly, the same effect was seen on $HCO_3^-$ secretion, but was not quite statistically significant, and 40 minutes after applying serosal sucrose $HCO_3^-$ secretion had been completely abolished (Figure 6.5F). The effects on electrophysiological parameters (Figures 6.5D and E) followed a similar trend to those shown opposite in the presence of mannitol.

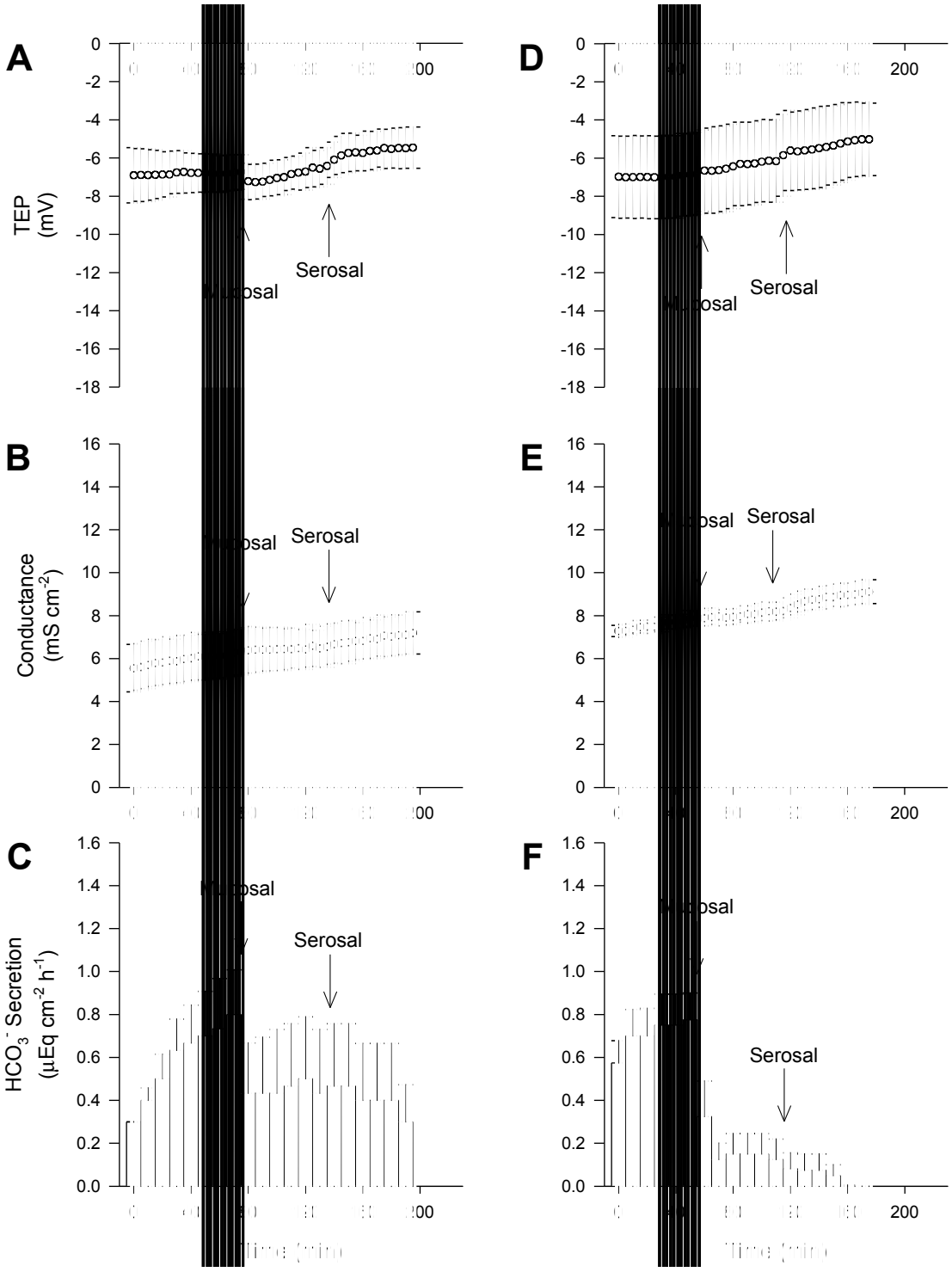



Figure 6.5: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^2$ $h^{-1}$) from the isolated anterior intestine of *F. heteroclitus* following the addition of 40 mOsm mannitol (Panels A to C), or 40 mOsm sucrose (Panels D to F) which were added first to the

mucosal, then to the serosal saline. Results are means ±SE based on n = 3 and 4 for treatments with mannitol and sucrose, respectively. Means with asterisks are significantly different from the preceding 30 minute contrd period, indicated by filled circles or bars ($P < 0.05$).

For some individuals $HCO_3^-$ was not merely reduced following the increase in mucosal osmolality but completely abolished. For example, in one case (Fish 69) the mucosal saline began to rapidly acidify with pH falling from 7.80 to 7.65 in the 15 minutes immediately following the addition of mannitol to the mucosal saline (Figure 6.6A). In addition, for Fish 72 and 74 the addition of sucrose had a similar effect (Figures 6.6B and C).

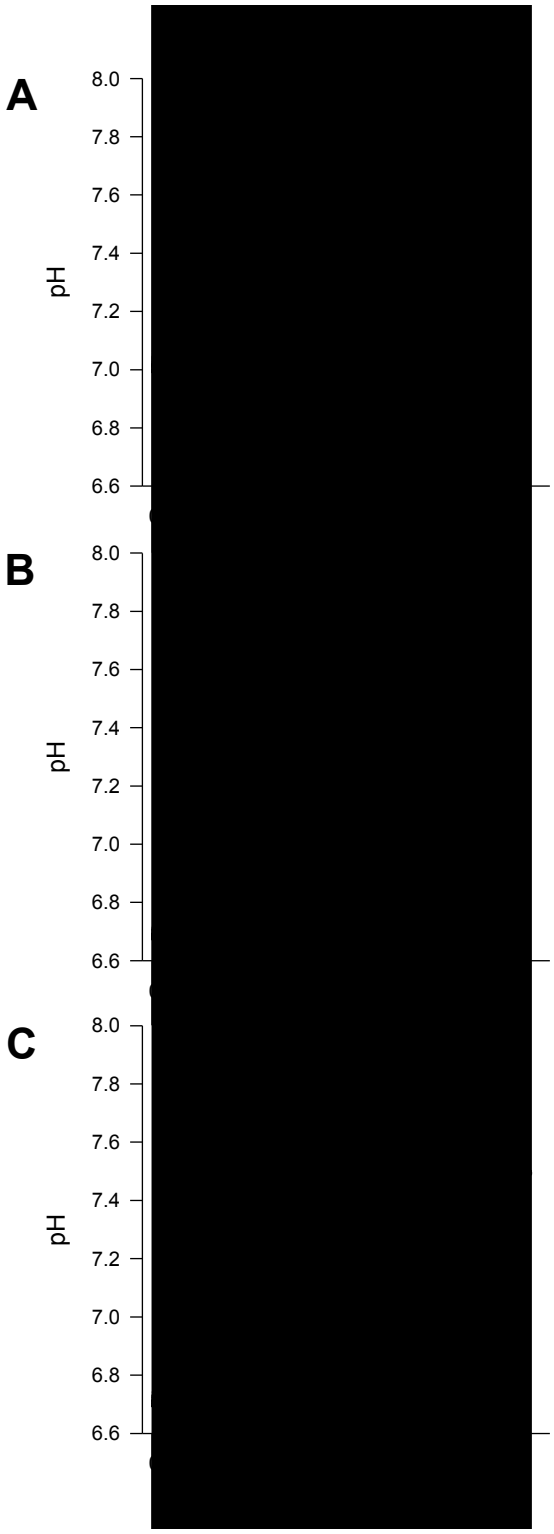



Figure 6.6: The pH of the mucosal saline logged over the course of an experiment which shows the increase in pH taking place at the beginning of an experiment as the tissue secretes $HCO_3^-$ up to pH 7.8 at which point the pH stat titration commences. The arrows

indicate the addition of either mannitol (Panel A – Fish 69) or sucrose (Panels B and C – Fish 72 and 74, respectively) to the mucosal and serosal salines and the subsequent effect on mucosal pH.

### 4.2.2 Source of $HCO_3^-$ secretion

Following the removal of serosal $HCO_3^-/CO_2$ there was no dramatic fall in $HCO_3^-$ secretion rates, instead they remained stable before beginning to decline slowly. After an hour $HCO_3^-$ secretion had fallen by only 25 % and similar to the data shown in Figures 6.5 and 6.6 subsequent addition of sucrose to the mucosal chamber all but abolished this secretion leading to acidification of the lumen (Figure 6.7).

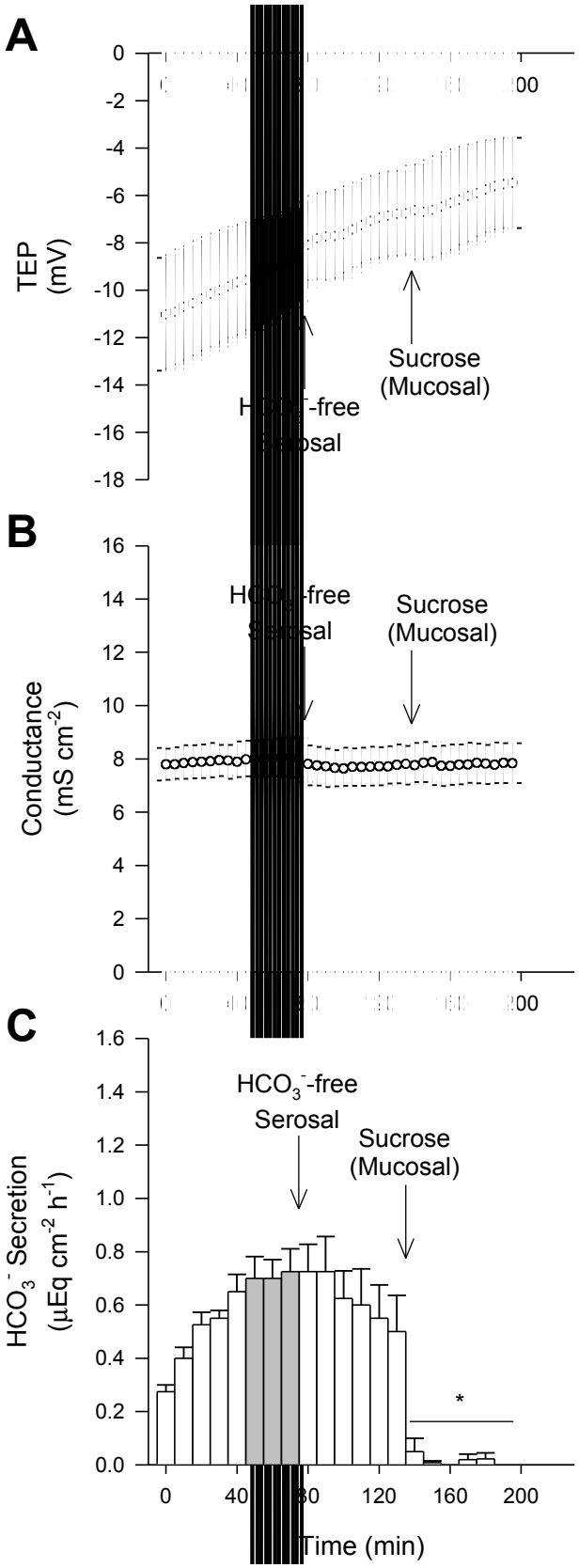

A
TEP (mV)
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
Sucrose (Mucosal)
B
Conductance (mS cm-2)
16
14
12
10
8
6
4
2
0
Sucrose (Mucosal)
C
HCO3- Secretion (µEq cm-2 h-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HCO3--free Serosal
Sucrose (Mucosal)
*
0
40
120
160
200
Time (min)

Figure 6.7: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^{-2}$ $h^{-1}$) from the killifish intestine after replacing the serosal saline, followed by the addition of 40 mOsm Sucrose to the mucosal saline. Results are means ±SE based on n = 4. Means with asterisks are significantly different from the preceding 30 minute control period, indicated by filled circles or bars ($P < 0.05$).

### 4.2.3 Basolateral $H^+$ secretion in relation to mucosal osmotic pressure

The rates of basolateral acid efflux were very high and in many cases more than double the rates of apical $HCO_3^-$ secretion seen previously. There appears to be little change in serosal proton secretion in response to increased mucosal osmolality by mannitol or sucrose (Figures 6.8C and F), nor were there any significant effects on TEP (Figures 8A and D) or conductance (Figures 6.8B and E).

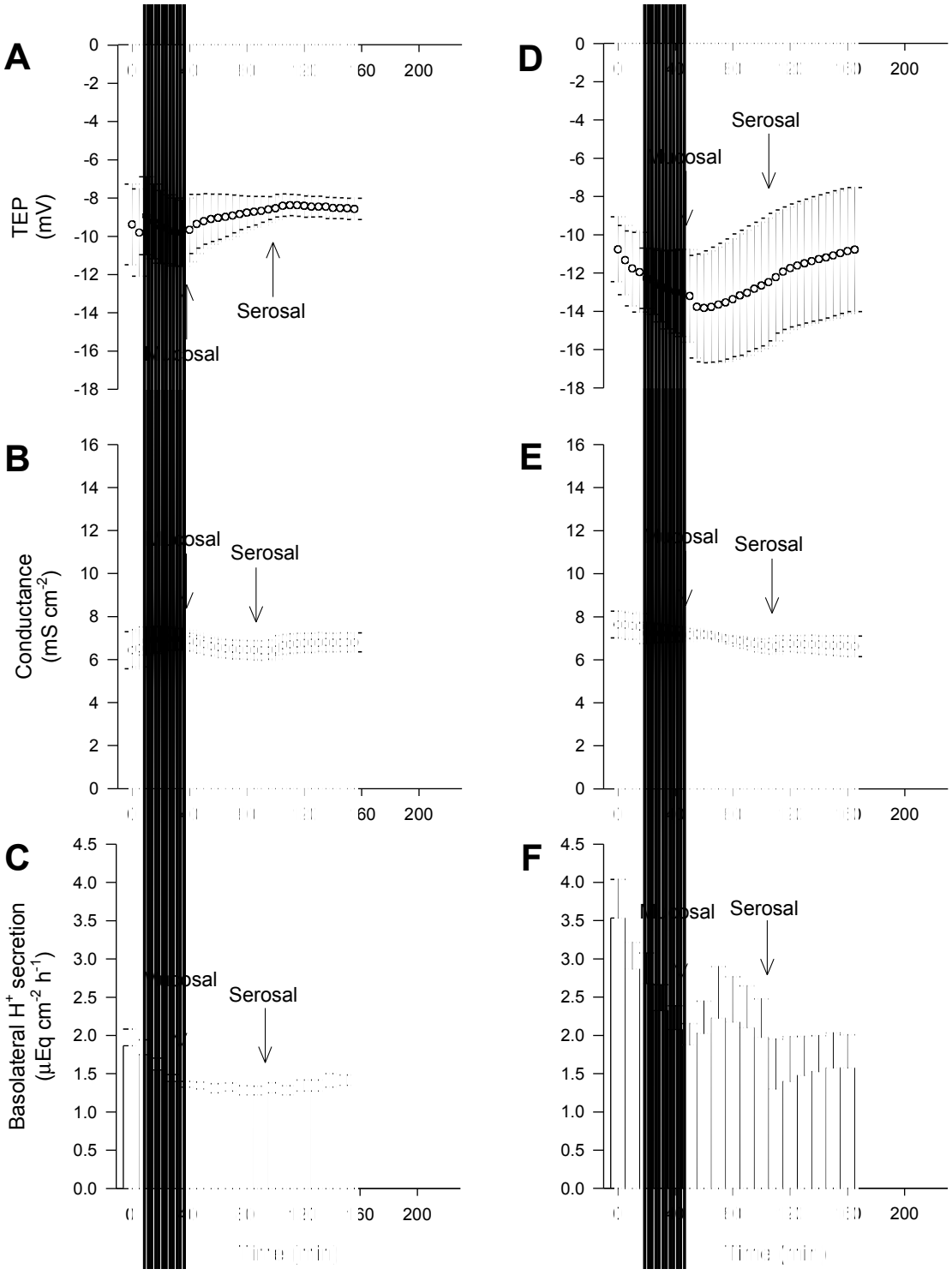



Figure 6.8: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and the serosal secretion of acidic equivalents (µEq $cm^{-2}$ $h^{-1}$) from the isolated anterior intestine of the killifish following the addition of 40 mOsm mannitol (Panels A to C), or 40 mOsm sucrose (Panels D to F) which were added

first to the mucosal, then to the serosal saline. Results are means ±SE based on n = 4 for both treatments. Means with asterisks are significantly different from the preceding 30 minute control period, indicated by filled circles or bars ($P < 0.05$).

### 4.2.4 Mediation of apical $HCO_3^-$ and $H^+$ secretion

DIDS was unexpectedly acidic and upon addition the mucosal saline dropped immediately taking anywhere from 40 to 90 minutes to recover to pH 7.8 and for titration to continue. Once the pH stat titration was resumed $HCO_3^-$ secretion took another 30 minutes to stabilise and was found to be significantly reduced by an average of 37 % (Figure 6.9C). This effect on $HCO_3^-$ was accompanied by a significant increase in conductance along with a reduction in TEP (Figures 6.9A and B). After 60 minutes, application of DIDS to the serosal saline had no further effect on secretion rates or electrophysiological parameters.

In an additional set of experiments with amiloride there was also a slight fall in pH, although not as severe as seen with DIDS, and the pH stat titration resumed swiftly with no effect on $HCO_3^-$ secretion (Figure 6.9F), nor any deleterious effects on the electrophysiogical properties of the tissue in general (Figures 6.9D and E). The effects of mucosal sucrose on $HCO_3^-$ secretion were not influenced by blocking $Na^+/H^+$ exchange, and although only n = 2, $HCO_3^-$ secretion by both preparations was immediately abolished.

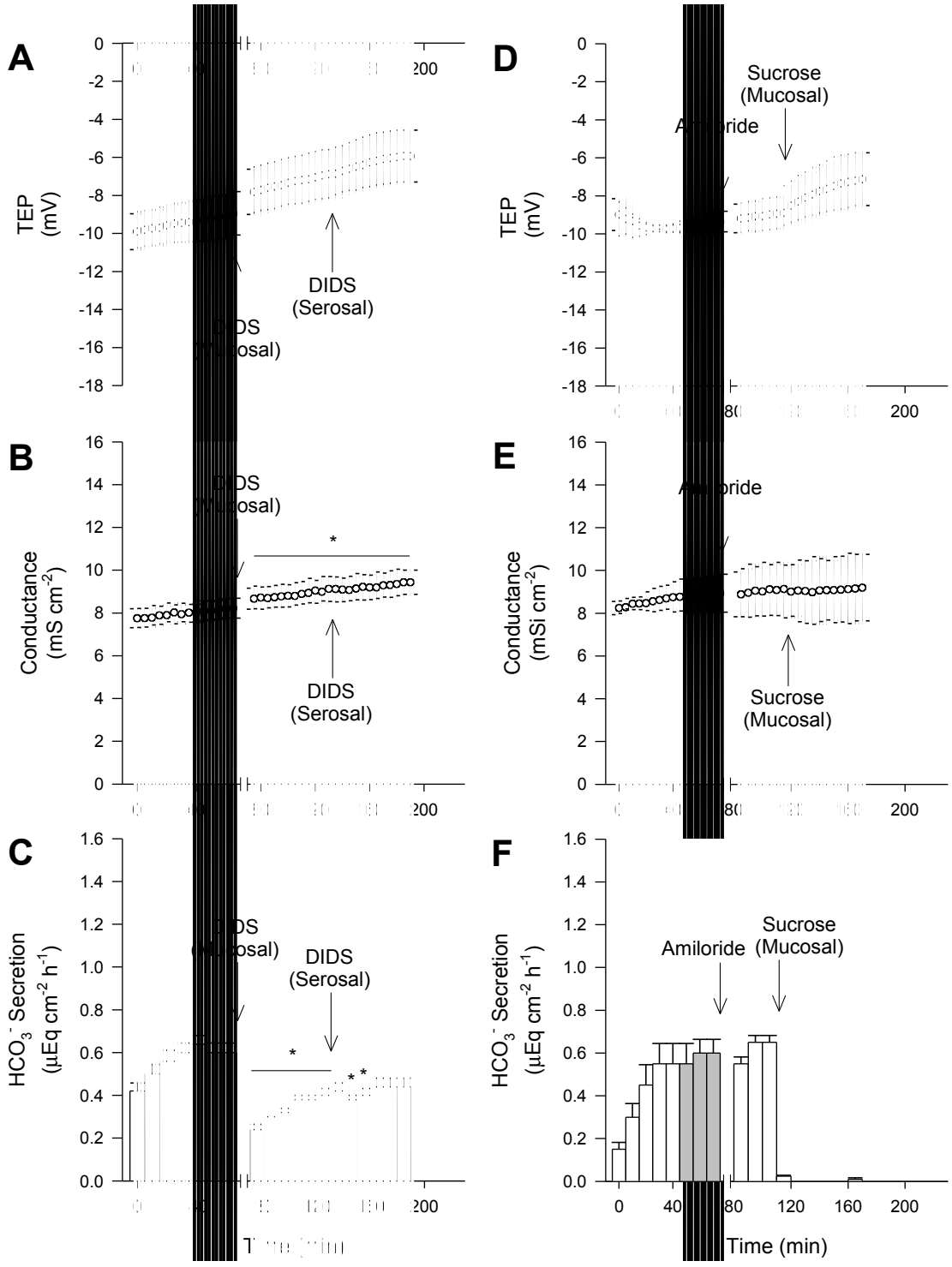



Figure 6.9: Simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^{-2}$ $h^{-1}$) from the isolated anterior intestine of the killifish following the application of mucosal DIDS ($10^{-4}$ M) and subsequent application of serosal DIDS ($10^{-4}$ M) (Panels A to C). Also, the

influence of mucosal amiloride ($10^{-4}$ M) followed by the addition of 40 mOsm sucrose to the mucosal saline (Panels D to F). Results are means ±SE based on n = 5 and 2 for the DIDS and amiloride treatments, respectively. Means with asterisks are significantly different from the preceding 30 minute contrd period, indicated by filled circles or bars ($P < 0.05$).

### 4.3 Buffer capacity of mannitol and sucrose

A separate set of titrations on the mucosal saline used in this study showed that the addition of mannitol did not significantly alter the buffer capacity of the saline. However, the presence of 40 mM sucrose required 10 % more base to reach pH 7.8 compared with mannitol or saline only (Figure 6.10).

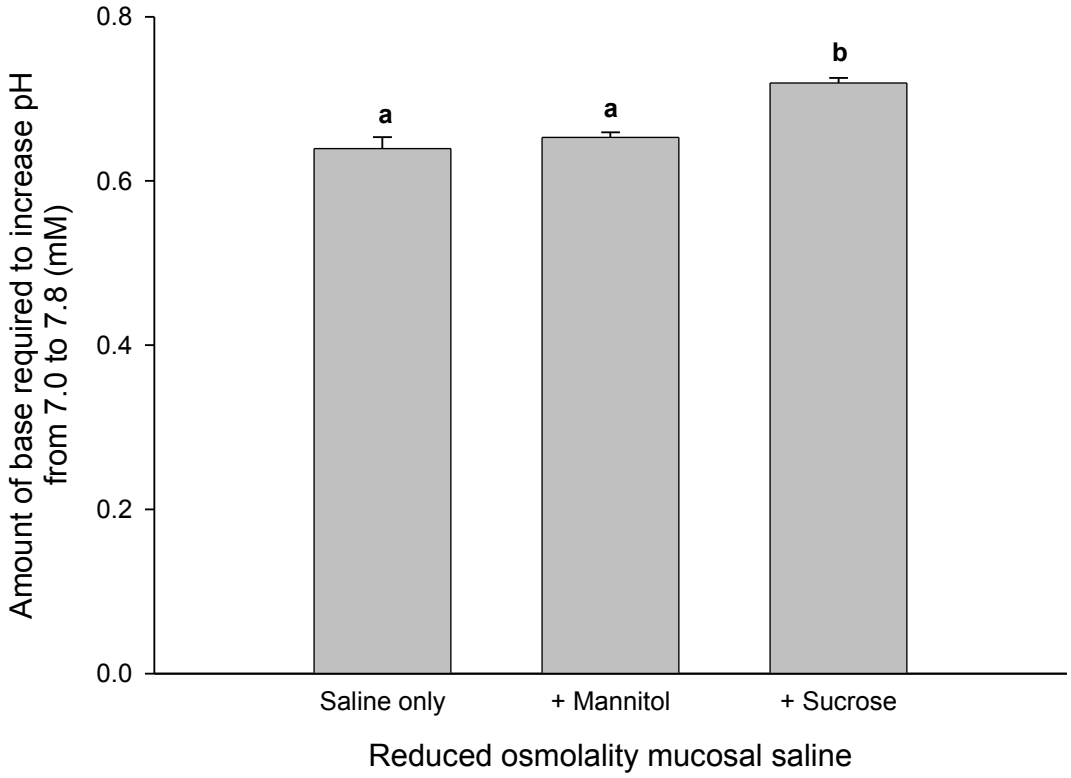



Figure 6.10: The mean (±SE) amount of base (mM) required to increase the pH of the reduced osmolality mucosal saline in order to determine any changes in buffer capacity following the addition of either 40 mM mannitol or sucrose. Means labelled with different letters indicate a significant difference ($P < 0.005$), n = 8 for each saline.

# 5. Discussion

## 5.1 Stimulation of $HCO_3^-$ secretion by $Ca^{2+}$

The killifish intestine displayed high rates of $HCO_3^-$ secretion, and based on all the measured control values from the present study this averaged 0.70 ±0.02 µEq $cm^{-2}$ $h^{-1}$ (n = 120) at 25 °C. This was approximately 3.5 times greater than the European flounder, at 12 °C using the same *in vitro* technique (Wilson *et al*., 2002). Yet in spite of this impressive secretion rate the epithelia was insensitive to the effects of $Ca^{2+}$ (and $Mg^{2+}$) following a 15 mM increase in the concentration of these divalent cations from 5 to 20 mM (Figure 6.2C). Before suggesting that this was a species-specific difference in the regulation of $HCO_3^-$ secretion it was first considered whether the ionic strength and osmotic pressure of the bathing media were able to influence the sensitivity of the putative calcium-sensing receptor (CaR).

## 5.2 Is $HCO_3^-$ secretion modulated by ionic strength and/or osmolality?

A comparison of the 'regular' mucosal saline with the mucosal saline used by Wilson *et al.* (2002), revealed a number of differences in their respective composition (Table 6.2). The $Na^+$ content was greater by 40 mM in the former (due to a higher concentration of NaCl), which also resulted in more $Cl^-$ being present. Overall, the total ionic strength and osmotic pressure of the saline in the present study was much higher. This was considered significant in terms of the predicted role of a CaR in the regulation of intestinal $HCO_3^-$ secretion (Wilson *et al*., 2002), since the activity of the receptor is known to be modulated by ionic strength (Quinn *et al*., 1998; Nearing *et al*., 2002; Fellner and Parker, 2004; Loretz *et al*., 2004).

Table 6.2: A comparison of the ionic composition of the 'regular' mucosal saline used in the present study with the mucosal saline used by Wilson *et al*. (2002). The concentration of each ion is given in mmol $l^{-1}$ and the units of osmolality mOsm $kg^{-1}$.

| Ion | Regular saline | Wilson *et al*. (2002) |
|---|---|---|

| | (Present study) | |
|---|---|---|
| $Na^+$ | 90.0 | 50.0 |
| $Cl^-$ | 135.0 | 110.0 |
| $K^+$ | 5.0 | 5.0 |
| $Ca^{2+}$ | 5.0 | 5.0 |
| $Mg^{2+}$ | 95.0 | 100.0 |
| $SO_4^{2-}$ | 80.0 | 77.5 |
| | | |
| Total | 410.0 | 347.5 |
| Osmolarity | 319 | 282 |

The next set of experiments employed a 'reduced ionic strength' mucosal saline, which had 90 mM fewer ions, compared with the 'regular' saline used previously (Table 6.1), and almost 30 mM lower than the saline used by Wilson *et al*. (2002). Yet again this failed to stimulate $HCO_3^-$ secretion, in fact there was a clear (but not quite significant) drop in secretion by almost one third (Figure 6.3C). Furthermore, there remains no clear explanation for the problems which frustrated experiments when applying $MgCl_2$ (Figure 6.3F). After inspecting the equipment and exhausting any potential technical causes, consideration was given to the tissue itself. In terms of active ion transport, TEP can be an (indirect) indicator of metabolic rate as the ability to maintain a TEP is dependent on the activity of the electrogenic $Na^+/K^+$ pump (Armstrong, 1987). However, in spite of depressed $HCO_3^-$ secretion rates there appeared no adverse effects on TEP (Figure 6.3D) or conductance (Figure 6.3E), and these parameters were comparable with values obtained from previous experiments. Another potential problem could have been related to the use of mannitol, which was included at a concentration of 80 mM to maintain the osmotic pressure of the saline at ~319 mOsm $l^{-1}$. While large concentrations of non-electrolytes, such as mannitol and sucrose, are known to interfere with electrode potentials, this is apparently only noticeable at molar concentrations, where TEP shifts in the positive direction by about 0.8 mV per 1 M mannitol (Barry and Diamond, 1970). With no obvious external or internal anomalies noted in the fish during dissection, these points suggested no inherent problems with the epithelium itself and the issue therefore seemed to solely concern the secretion of $HCO_3^-$. Also, these problems did not manifest themselves in prior experiments using the same 'reduced ionic strength' mucosal saline (Figures 6.3A and B). Mannitol is also known to donate a proton in aqueous solution, and is therefore considered weakly acidic (Gaidamauskas *et al*., 2005), which may have buffered secreted $HCO_3^-$. However, this was also considered an unlikely scenario as prior experiments revealed that

the $HCO_3^-$ secretion rate was unaffected (Figure 6.3C), and this conclusion was further supported by confirmation that mannitol does not alter the buffer capacity of the mucosal saline (Figure 6.10).

A third and final manipulation of the mucosal saline was one of reduced ionic strength but with no osmotic compensation, and therefore virtually identical to the saline used by Wilson *et al*. (2002). Interestingly, as quickly as the above described problem had developed it disappeared after switching to this saline, and $HCO_3^-$ secretion rates were restored. However, there were yet again no effects of $Ca^{2+}$ (or $Mg^{2+}$) on the rate of $HCO_3^-$ secretion (Figure 6.4C and F).

### 5.2.1 Other effects on epithelial ion transport

Regardless of the bathing medium the addition of either $CaCl_2$ or $MgCl_2$ had similar effects on electrophysiological measurements displaying an immediate, significant decrease in TEP (i.e. becoming more negative). Even though it was not possible to determine the nature of this change it was considered likely to represent an increase in net $Cl^-$ absorption from mucosa to serosa, due to the increased availability of $Cl^-$ following the addition of either 15 mM $CaCl_2$ or $MgCl_2$. This raised the concentration of mucosal $Cl^-$ by 30 mM, and depending on the mucosal saline employed amounted to an 18-25 % increase in mucosal $Cl^-$, fitting in well with the overall average increase in TEP of 20 ±2 % across treatments. Accompanying this change in TEP was a similar increase in transepithelial conductance ($G_t$), an indicator of the ionic permeability of the epithelia (Horowicz *et al*., 1978). Conductive pathways for ions through epithelia can be either transcellular or paracellular. In epithelia such as the intestine which are typically characterised as 'leaky' almost all of the measured $G_t$ represents ionic permeation *via* the paracellular pathway, and can be as much as 80-96 % in the marine teleost intestine (Loretz, 1995), within the range reported for mammals and other vertebrates (Powell, 1981; Armstrong, 1987). It was not possible to determine the nature of this increase in $G_t$, but it may represent an increase in basolateral $Cl^-$ conductance (concomitant with the assumption of increased $Cl^-$ absorption, based on TEP).

### 5.2.2 A role for the CFTR?

In the seawater-adapted killifish the observed relationship between $Cl^-$ fluxes and $G_t$ by the intestine suggests that a substantial portion of $Cl^-$ flux is indeed conductive (Marshall *et al.*, 2002), and is supported by the identification and functional localisation of a cystic fibrosis transmembrane conductance receptor (CFTR) to the apical and basolateral membranes of the posterior intestine in *F. heteroclitus* (Singer *et al.*, 1998; Marshall *et al.*, 2002). The CFTR is a channel forming protein that primarily transports $Cl^-$ and is expressed in a range of epithelia including the intestine, lungs, skin and pancreas (Fuller and Benos, 1992). Mutations of the CFTR gene are widely believed to result in abnormal ion and fluid transport giving rise to the disease cystic fibrosis (Fuller and Benos, 1992). Thus, the observed increase in $G_t$ may therefore represent an upregulation of $Cl^-$ conductance *via* a chloride transporting channel such as the CFTR. In contrast to the killifish, no similar effects on TEP or $G_t$ were detected in the flounder intestine (Wilson *et al.*, 2002). However, Wilson and co-workers only added 5 mM $CaCl_2$ as opposed to 15 mM in the present study.

On reviewing the data shown in Figures 6.2 to 6.4, there was no evidence of a role for mucosal $Ca^{2+}$ in the stimulation of $HCO_3^-$ secretion by the killifish intestine *in vitro*. Based on the predicted involvement of a CaR, manipulations of ionic strength and osmolality of the surrounding media did not influence the response of the tissue suggesting that, unlike the flounder, there may well be a different mechanism behind the stimulation of intestinal $HCO_3^-$ secretion in the killifish.

**5.3 Effects of mucosal hyperosmolarity and the role of cell volume regulation**

The addition of either 15 mM $CaCl_2$ or $MgCl_2$ not only increased the ionic strength of the mucosal saline relative to the serosal, but also imposed a relatively large osmotic gradient across the tissue, presenting a significant challenge to cell volume control drawing water out of the epithelia. Changes to cell volume induced by alterations to extracellular osmolality (causing either shrinking or swelling due to the associated movement of water) can jeopardise the structural and functional integrity of the cell. Exposure to anisoosmotic media therefore results in a rapid (within minutes) recruitment of additional ion transport mechanisms to help correct for these potentially damaging volume changes. These processes are termed the regulatory volume increase (RVI) and regulatory volume decrease

(RVD) in relation to the influence of hyper- and hypoosmotic media, respectively (Lang *et al*., 1998; O'Neill, 1999).

Of particular relevance to the present study following the increase in mucosal osmolarity was the RVI, which is typically mediated by the activation of $Na^+$-$K^+$-$2Cl^-$ cotransport (NKCC) and also $Na^+/H^+$ exchange (NHE) coupled to parallel $Cl^-/HCO_3^-$ exchange. Both mechanisms increase the net influx of NaCl which will be followed by osmotically obliged water, thus counteracting cell shrinkage (Lang *et al*., 1998; O'Neill, 1999). Recently, Grosell *et al*. (2007) predicted for the rainbow trout that increases in mucosal osmolarity may trigger a shift in $H^+$ secretion by intestinal epithelial cells, from basolateral to apical, which would titrate luminal $HCO_3^-$ (to $CO_2$) thus helping reduce luminal osmotic pressure (discussed previously in Chapter 4, Section 5.2.2). This is a particularly intriguing suggestion for the estuarine killifish also, as it is likely to experience varying fluid osmolalities within the intestine on a regular basis. However, following the addition of $CaCl_2$ (in an attempt to stimulate $HCO_3^-$ secretion), which increased the osmolarity of the mucosal chamber by approximately 40 mOsm $l^{-1}$, there was no significant reduction in $HCO_3^-$ secretion, which may well have been observed if mucosal $HCO_3^-$ were being titrated by $H^+$ as part of the RVI.

When the osmolarity of both salines bathing the isolated freshwater eel (*Anguilla anguilla*) intestine were simultaneously increased from 290 to 315 mOsm $l^{-1}$ (using mannitol) ion transport displayed a biphasic response (Lionetto *et al*., 2001). The first phase was a rapid, transient increase approximately 10 minutes in duration, correlating with histological observations of cellular shrinkage. This was followed by a sustained second phase, recognised as the RVI, and involved increased $Cl^-$ transport *via* the recruitment of apical NKCC and basolateral $Cl^-$ channels, which reached a steady state after 30-45 minutes. Notably, these observations appear similar to the rapid changes in TEP and $G_t$ in the present study following the addition of either $CaCl_2$ or $MgCl_2$, and thus lend additional support to the earlier prediction that the changes to epithelial ion transport represent the movement of $Cl^-$ from mucosa to serosa (Section 5.2).

However, increasing mucosal osmolarity (by 40 mOsm $l^{-1}$), using either mannitol or sucrose, did not have the same effect as the $Cl^-$ salts but instead resulted in a rapid and dramatic reduction in $HCO_3^-$ secretion, with no significant changes to TEP or $G_t$ (Figure 6.5). Subsequently raising the osmolarity of the serosal saline, effectively removing the

transepithelial osmotic gradient, did not restore $HCO_3^-$ secretion rates. Even though sample sizes within each of these data sets were rather small (n = 3 and 4), and as such reduced the likelihood of detecting any statistically significant differences, the effect on $HCO_3^-$ secretion was convincing. In fact, for some individuals the pH stat titration was abolished after the saline pH fell below 7.8, and the mucosal saline began to slowly acidify (Figure 6.6). These results were very intriguing and offered tentative support to the prediction that the coupling of apical $HCO_3^-$ with $H^+$ secretion was indeed activated folowing mucosal hyperosmolality. Curiously, this response was only apparent when the change in osmolarity was induced by (largely) impermeant solutes (mannitol and sucrose) and not by ionic solutes ($CaCl_2$ and $MgCl_2$).

### 5.3.1 The source of $HCO_3^-$

Experiments employing a $HCO_3^-/CO_2$-free serosal saline, demonstrated that approximately 75 % of secreted $HCO_3^-$ by the killifish intestine was derived from the intracellular hydration of $CO_2$ (Figure 6.7). This was contrary to the initial prediction that secretion was largely dependent on a serosal supply of $HCO_3^-/CO_2$ and also the conclusion by Lionetto *et al*. (2001) that serosal $HCO_3^-$ was crucial for the response to hyperosmolarity, since under $HCO_3^-/CO_2$-free serosal conditions the epithelial response to hyperosmolarity was lost by the freshwater eel. This was clearly not the case for the seawater-adapted killifish, in fact the absence of serosal $HCO_3^-/CO_2$ seemed to attenuate the response as apical $HCO_3^-$ secretion was all but abolished following the addition of mucosal sucrose (Figure 6.7C).

### 5.3.2 Basolateral $H^+$ secretion

If a shift in the direction of $H^+$ secretion from basolateral to apical were indeed responsible for this reduction in $HCO_3^-$ secretion then it was not evident from the measurements of basolateral $H^+$ secretion (Figure 6.8). Although these latter experiments into serosal $H^+$ secretion were potentially useful they were not without problems. There are two major points to be highlighted regarding these particular experiments. Firstly, more time should have been permitted for the preparation to settle before applying either mannitol or sucrose. In contrast to the pH stat titration of $HCO_3^-$ secretion into the mucosal saline, pH stat titration of the serosal saline would begin immediately and shortly thereafter the first applications of mucosal mannitol and sucrose were applied. As seen from the associated

electrophysiological measurements and acidification rates in Figure 8 these parameters had not completely stabilised. Secondly, there would also have been a substantial pH gradient across the tissue for at least the first hour of these experiments which may have influenced $H^+$ secretion. While the serosal saline was fixed at pH 7.8, the initially unbuffered mucosal saline started at a pH between 6.4 and 7.1, and during a typical pH stat titration of the mucosal saline it would often take an hour, perhaps longer, for the epithelia to secrete sufficient $HCO_3^-$ equivalents to reach pH 7.8.

In spite of these problems, the rate of $H^+$ equivalents secreted into the serosal saline was more than double the recorded rate of mucosal $HCO_3^-$ secretion, with potential reasons for this having been discussed previously (Chapter 4, Section 5.3.2). Recording such high rates may have made it difficult to detect a shift in the direction of $H^+$ secretion from basolateral to apical in response to mucosal hyperosmolarity. However, based on the reduction in pH following the addition of mannitol and sucrose shown in Figure 6.6, the rate of mucosal acidification would have been approximately 0.015 $\mu$Eq $cm^2$ $h^{-1}$, together with an anticipated 0.45 $\mu$Eq $cm^2$ $h^{-1}$ reduction in serosal $H^+$ (based on the decrease in $HCO_3^-$ secretion rate shown in Figures 6.5C and F) it should have been possible to detect this predictable fall in serosal $H^+$ secretion of approximately 25 %.

**5.4 The response of the killifish intestine to mucosal hyperosmolarity**

To help summarise the discussion so far, Figure 6.11 presents a direct comparison of the differing responses of the seawater-adapted killifish intestine following increases in mucosal osmolarity by permeable, ionic solutes ($CaCl_2$ and $MgCl_2$) and the non-ionic, impermeable osmolytes (mannitol and sucrose). The distinguishing features are the differing responses of TEP and $HCO_3^-$ secretion between treatments (Figures 6.11A and C). Following the addition of the $Cl^-$ salts there was a rapid change in TEP (Figure 6.11A), presumably a reflection of elevated apical NaCl cotransport and basolateral $Cl^-$ conductance (Lionetto *et al.*, 2001; Marshall *et al.*, 2002; Lionetto and Schettino, 2006; Hoffman *et al.*, 2007). Whereas, mucosal hyperosmolarity induced by mannitol and sucrose, would have left the concentrations of extracellular ions unchanged. Thus, the driving force for activation of an ionic influx (followed by water) as part of the RVI will be greatly reduced compared with either $CaCl_2$ or $MgCl_2$. Instead, there was a dramatic reduction in $HCO_3^-$ secretion (Figure 6.11C), associated with a biphasic change in total

ionic flux. The latter was characterised by a small, rapid decrease in TEP immediately after inducing hyperosmolarity and lasted approximately 10 minutes. This was followed by a sustained and gradual increase over the proceeding 40 minutes (Figure 6.11A). In contrast, the second phase of the RVI by the freshwater eel intestine to a similar hyperosmotic challenge (by simultaneous addition of mannitol to both sides of the epithelium) revealed a sustained decrease in TEP (Lionetto *et al.*, 2001). One possibility accounting for this discrepancy in response compared with the seawater-adapted killifish was the likelihood that apical $HCO_3^-$ secretion by the freshwater eel intestine would be inherently low (Wilson, 1999; Wilson *et al.*, 2002; Grosell, 2006), and therefore unavailable to the RVI.

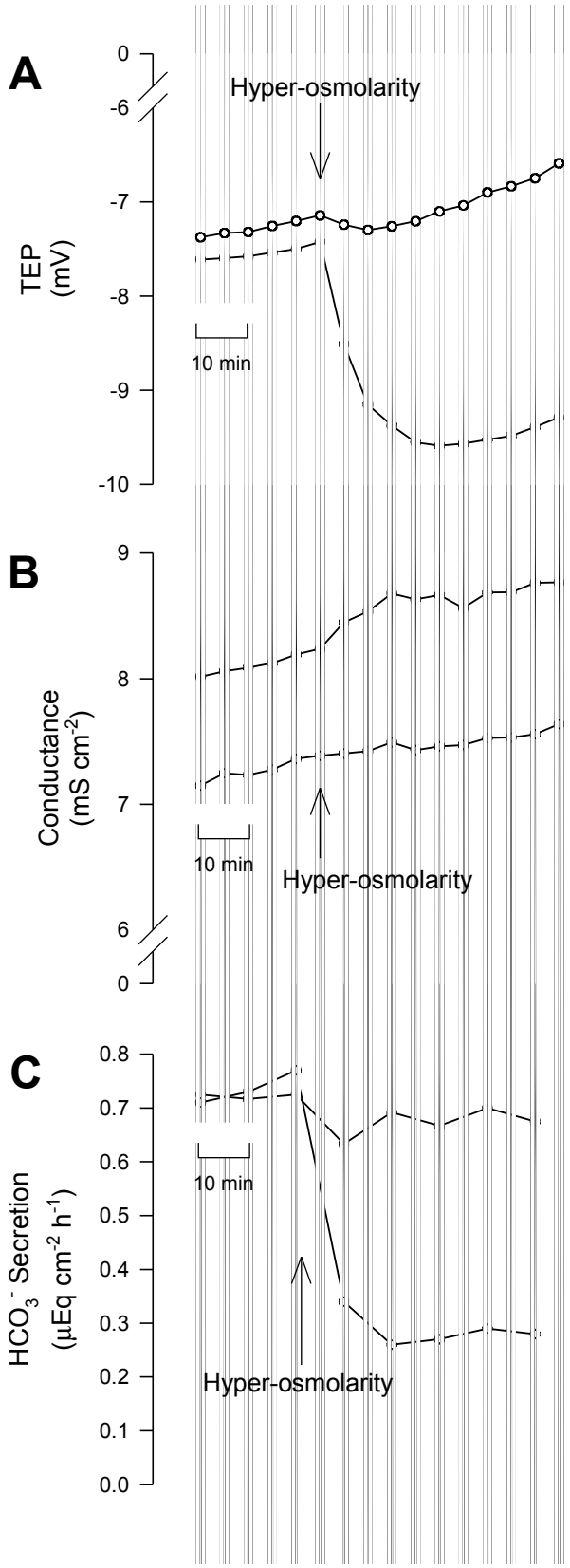

A
0
-6
-7
-8
-9
-10
TEP (mV)
Hyper-osmolarity
10 min
B
9
8
7
6
0
Conductance (mS cm-2)
10 min
Hyper-osmolarity
C
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HCO3- Secretion (µEq cm-2 h-1)
10 min
Hyper-osmolarity

Figure 6.11: A comparison of the simultaneous measurements of transepithelial potential, TEP (mV), transepithelial conductance, G (mS $cm^{-2}$) and $HCO_3^-$ secretion (µEq $cm^{-2}$ $h^{-1}$) from the killifish intestine responding to a 40 mOsm $l^{-1}$ increase in mucosal osmolarity induced by ionic, permeable osmolytes ($CaCl_2$ and $MgCl_2$), denoted by the black circles (n = 12), or impermeant solutes (mannitol and sucrose), shown by the open circles (n= 10). Data are displayed as means values from all experiments employing the 'reduced osmolality' mucosal saline (~282 mOsm $l^{-1}$).

### 5.4.1 Mediation of apical $HCO_3^-$ and $H^+$ secretion

Blocking apical $Na^+/H^+$ with the non-specific inhibitor amiloride did not affect the abolition of $HCO_3^-$ secretion on application of sucrose (Figure 6.9F). Even though the sample size was only n = 2 the result was convincing and implies that the proposal of mucosal acidification following hyperosmotic challenge did not necessarily involve the recruitment of $Na^+/H^+$ exchange. One further option that could have been explored at this point in the study was to try blocking the $H^+$-ATPase with bafilomycin. However, as time was running short for this visit it was decided to explore whether the reduction in $HCO_3^-$ secretion could due to an inhibition of anion exchange as opposed to titration of secreted $HCO_3^-$, which would also afford the opportunity to simultaneously provide evidence for the involvement of $Cl^-/HCO_3^-$ exchange by the killifish intestine.

The application of mucosal DIDS significantly reduced $HCO_3^-$ secretion, thus providing evidence for the contribution of apical $Cl^-/HCO_3^-$ exchange. It was surprising to find that DIDS (and amiloride) dissolved in DMSO were so acidic, consequently holding up the pH stat titration. In spite of this $HCO_3^-$ secretion continues and within 30 minutes of reaching pH 7.8, and restart of the pH stat titration, secretion rates had stabilised to 63 % of control values (Figure 6.9C). Subsequent application of DIDS to the serosal saline did not influence apical $HCO_3^-$ secretion and to some extent supports the earlier conclusion that the source of $HCO_3^-$ is largely endogenous arising from $CO_2$ hydration (Figure 6.7C). Interestingly, mucosal $HCO_3^-$ secretion was not abolished in response to DIDS which would suggest there may be additional mechanisms of $HCO_3^-$ transport. The fact that the mucosal saline was able to recover to pH 7.8 after falling to pH 6.15 ±0.15 (n= 5), and the secretion rate continued to increase for a further 30 minutes upon reaching 7.8 before

stabilising (Figure 6.9C) suggested that there was a potential compensatory mechanism of $HCO_3^-$ secretion.

### 5.4.2 The mechanism of $HCO_3^-$ secretion *in vitro*

It has previously been recognised that DIDS does not produce a complete inhibition of $HCO_3^-$ secretion by intestinal epithelia from a number other marine teleosts *in vitro* (Dixon and Loretz, 1986; Ando and Subramanyam, 1990; Grosell and Jensen, 1999; Grosell *et al*., 2001), which have been considered the result of the relatively low concentrations of DIDS used (Grosell, 2006), the topography of the intestine (Grosell and Jensen, 1999) and/or insensitivity of the particular $Cl^-/HCO_3^-$ exchanger isoform to DIDS (Grosell *et al*., 2001; Romero *et al*., 2004). An additional consideration has been the potential for conductive $HCO_3^-$ exit (Dixon and Loretz, 1986). For example, in these pH stat experiments where the mucosal saline is initially unbuffered and the subsequent $HCO_3^-/CO_3^{2-}$ content low, along with a net negative cytosol, there will be a large electrochemical gradient for conductive $HCO_3^-$ movement across the apical membrane. Blocking apical $Cl^-/HCO_3^-$ exchange would lead to a build up of intracellular $HCO_3^-$ and further fuel this gradient for apical exit. This can be demonstrated by applying the Nernst equation which describes the equilibrium potential, E (mV) required to sustain a given ion gradient across an epithelium and is given as:

$$E = (RT) / (zF) \times Log_{10} (X_o / X_i) \qquad (2)$$

Where, R is the universal gas constant (8.31441 J $K^{-1}$ $mol^{-1}$), T is the absolute temperature in Kelvin (25 °C = 298 K), z is the valence of the ion (for $HCO_3^-$ this is -1), F is Faradays constant (96484.6 coul $mol^{-1}$), $X_o$ is the concentration of the ion in the outer solution (mucosal saline) and $X_i$ the concentration of the ion in the inner solution (serosal saline). Based on the mean secretion rate of 0.7 µEq $cm^{-2}$ $h^{-1}$ and the volume of the chamber (1.6 ml), the concentration of $HCO_3^-$ in the mucosal saline was taken as 0.1 mM, with 8 mM in the serosal saline (Table 6.1), the equilibrium potential was calculated as 48.9 mV compared with an average measured TEP of -9.4 mV indicating a substantial gradient for $HCO_3^-$ secretion by conductive pathways. In contrast, *in vivo* conditions where mucosal concentrations may be in excess of 100 mM, the calculated equilibrium potential would be

around -28 mV, much lower than the TEP measured here. Secondarily active $Cl^-/HCO_3^-$ exchange will therefore become increasingly important as the concentration of $HCO_3^-$ increases in the gut lumen (Grosell *et al.*, 2001; Grosell *et al.*, 2005; Grosell, 2006). Furthermore, it has been observed that blocking anion exchange with DIDS leads to compensatory $HCO_3^-$ secretion *via* anion conductance (Novak, 2000) which could help explain the observed reduction in TEP and significant increase in $G_t$ (Figures 6.9A and 6.9B, respectively). While DIDS is widely used as a pharmacological blocker of anion exchangers and $Cl^-$ channels, the CFTR is insensitive to DIDS (Marshall *et al.*, 2002) and as this channel is also capable of transporting $HCO_3^-$ it will therefore be a potential conductive pathway accounting for the sustained mucosal $HCO_3^-$ secretion observed in Figure 6.9C. It is important to remember that there are also a number of additional effects of DIDS including the activation of cation conductance in *Xenopus* oocytes (Diakov *et al.*, 2001; Stumpf *et al.*, 2006) which could explain some of the observed changes in TEP and $G_t$. In these experiments the dramatic reduction, and in some case abolition of $HCO_3^-$ secretion, in response to mucosal hyperosmolarity cannot be attributed to the inhibition of anion exchange.

### 5.4.3 Physiological significance

There is a clear distinction in the response of the intestine to hyperosmolarity induced by $Cl^-$ salts and impermeant osmolytes (Figure 6.11). This begs the question, why are there such different responses to the same increase in osmotic pressure and what would be their physiological significance? It would seem the answer lies with salinity adaptation, as an estuarine species the killifish must be capable of readily augmenting its osmotic and ionic homeostasis. The application of $CaCl_2$ and $MgCl_2$ increased the concentration of luminal $Cl^-$ (as well as $Ca^{2+}$ and $Mg^{2+}$), and osmolarity, conditions that would not be dissimilar to what the gastrointestinal tract may experience when environmental salinity increases and drinking rate is consequently elevated (Chapter 1, Section 2.1). Even though $HCO_3^-$ secretion was not stimulated in response to $Ca^{2+}$ as expected, intestinal $Cl^-$ absorption increased (discussed in Section 5.2.2) which *in vivo* would inevitably contribute to increased levels of $Cl^-$ in the blood and an associated increase in plasma osmolarity. For example, when killifish were rapidly transferred from freshwater to full strength seawater the osmotic pressure of the blood plasma increased (by as much as 65 mOsm $kg^{-1}$), in turn

this hyperosmotic shock activated $Cl^-$ secretion by the gills (Zadunaisky *et al.*, 1995), and this has subsequently been associated with elevated expression of CFTR in the intestine and mitochondria-rich cells following similar salinity transfer (Singer *et al.*, 1998).
In contrast, mucosal hyperosmolarity induced by the non-ionic osmolytes (mannitol and sucrose) does not lead to a concomitant increase in intestinal $Cl^-$ absorption. Although these investigations are far from complete, the proposal that apical $H^+$ secretion is upregulated, acidifying the lumen and titrating mucosal $HCO_3^-$, consequently reducing luminal osmotic pressure is an appealing hypothesis worthy of additional study. Since the killifish is stomachless, the anterior intestine (used in these studies), will be the main site of digestion receiving pancreatic and biliary inputs and therefore experience large shifts in osmotic pressure during digestion, as well as coping with ingested seawater. It is worth noting that although mannitol and sucrose are both carbohydrates, it was very unlikely that the reduction in $HCO_3^-$ secretion resulting from this specific hyperosmotic challenge was a digestive response. For example, gastric secretions have been shown to increase the buffer capacity of chyme entering the intestine by 33 % in human subjects (Fordtran and Walsh, 1973). However, Babkin and Bowie (1928) have shown that the killifish does not undertake acidic digestion. Furthermore, the use of isolated sections of intestine by these experiments means that any pancreatic or biliary inputs will be absent, and also any digestive enzymes secreted by the intestinal cells themselves are unlikely to be significant (Fänge and Grove, 1979). In addition, only sucrose significantly altered buffer capacity (Figure 6.10), yet was clearly unable to account for the sustained rates of mucosal acidification shown in Figure 6.6.

# Chapter Seven

## Is there a role for the calcium-sensing receptor in the regulation of ion and fluid transport by the marine teleost intestine?

## 1. Summary

The functional implications of the calcium-sensing receptor (CaR), particularly in relation to gastrointestinal physiology, reveals a variety of potential roles from nutrient sensing to modulating ion and fluid transport, immune function and also epithelial renewal, yet for teleosts there is almost no direct evidence in support of these ideas. Activation of the CaR by its primary ligand, $Ca^{2+}$ is thought to be responsible for the stimulation of $HCO_3^-$ secretion as demonstrated by the intestine of the seawater-adapted European flounder *in vitro* and *in vivo*. The aim of the present study was to investigate a potential role for the CaR in the regulation of ion and fluid transport by the teleost intestine. Considering the possibility that the receptor is modulated by extracellular ionic strength, using the *in vitro* paired gut sac technique with a reduced ionic strength mucosal saline resulted in an overall decrease in the rate of $HCO_3^-$ secretion in response to elevated mucosal $Ca^{2+}$, while fluid absorption was reversed and preparations displayed net secretion. Similarly, lowering the ionic strength and osmolality of the mucosal saline had identical effects on ion and fluid transport in the presence of 20 mM $Ca^{2+}$ compared to controls. Mucosal application of gadolinium ($Gd^{3+}$) and neomycin, both recognised agonists of the CaR, did not produce responses in terms of $HCO_3^-$ secretion and fluid transport that were fully consistent with the influence of $Ca^{2+}$. Upon analysing the thermodynamics of the gut sac system it became clear that as $HCO_3^-$ accumulated within the mucosal saline over the course of an incubation the electrochemical potential for secondarily active $Cl^-/HCO_3^-$ exchange would be substantially diminished. By contrast, when using the Ussing chamber with pH stat titration, a favourable gradient for $HCO_3^-$ secretion was constantly maintained. Taking into account potential limitations on intracellular $HCO_3^-$ production, and the influence of unstirred layers within the static mucosal saline, it was subsequently argued that the gut sac was an inappropriate technique for measuring increases in $HCO_3^-$ secretion *in vitro*. Having already presented compelling evidence of a role for $Ca^{2+}$ in the stimulation of $HCO_3^-$ secretion, $CaCO_3$ production and fluid transport *in vivo*, addition of $Gd^{3+}$ and neomycin to the perfusion salines did not significantly influence intestinal $HCO_3^-$ secretion or fluid absorption compared to controls. It was suggested that the role of the CaR would in fact be largely redundant *in vivo*, instead $HCO_3^-$ secretion would be determined by two factors. Firstly, the ability to generate high levels of intracellular $HCO_3^-$, and secondly the creation

of a localised gradient for apical $HCO_3^-$ exit as it is consumed in the formation of $CaCO_3$. Alternatively, under *in vitro* conditions, where $CaCO_3$ would be unlikely, the presence of additional $Ca^{2+}$ stimulates $HCO_3^-$ secretion (presumably *via* a CaR) to encourage $CaCO_3$ production and removal of the excess $Ca^{2+}$. While this would appear true for the Ussing chamber technique, it was disappointing to learn that the inability to observe this same process, and the effects on ion and fluid transport using gut sacs, were most probably undermined by the constraints of this technique.

## 2. Introduction

There is growing evidence that the calcium-sensing receptor (CaR) is likely to influence a number of different functions in the teleost intestine, including the rate of $HCO_3^-$ secretion, where the receptor is thought to mediate this process by signalling changes in luminal $Ca^{2+}$ concentrations (Wilson *et al*., 2002). It was hypothesised that elevated mucosal $Ca^{2+}$ would stimulate $HCO_3^-$ secretion, and possibly net $Cl^-$ absorption (*via* $Cl^-/HCO_3^-$ exchange), and in turn drive additional fluid absorption. Unfortunately, the *in vitro* gut sac experiments described previously have so far been unsuccessful in resolving the effect of mucosal $Ca^{2+}$ on intestinal $HCO_3^-$ secretion. From analysis of the data presented in Chapter 4, elevated $Ca^{2+}$ appeared to reduce net NaCl and fluid transport in the anterior and mid portions of the intestine (Section 4.1). Furthermore, in the absence of serosal $HCO_3^-/CO_2$ these transport processes were abolished across all sections of the gut (Section 4.3). It was subsequently argued that a CaR might be involved in mediating this rather unexpected response (Section 5.3.4).

Interestingly, a reduction in NaCl and fluid transport in the presence of elevated luminal $Ca^{2+}$ is contrary to the demonstration of increased $HCO_3^-$ secretion both *in vitro* (Wilson *et al*., 2002) and *in vivo* (Wilson *et al*., 2002; Chapter 5). With the possibility that activation of the CaR can mediate $HCO_3^-$ secretion (Wilson *et al*., 2002), as well as net NaCl transport (Chapter 4), and consequently water absorption by the teleost intestine, the following study set out to try and demonstrate a functional role for the receptor. A brief review of the literature on the CaR in fish, with particular emphasis on the gut, reveals that in spite of its

name the receptor is likely to have a variety of roles, as it has the capacity to recognise and respond to a number of different physiological ligands.

**2.1 Calcium homeostasis**

Teleosts have successfully demonstrated adaptation to a range of ambient $Ca^{2+}$ concentrations, from ion poor freshwater, containing as little as 0.001 mM $Ca^{2+}$ (Gonzalez *et al.*, 2005), to seawater (~10 mM), and beyond for species at hyper-salinities such as the killifish, *Fundulus heteroclitus* (Griffith, 1974). The maintenance of stable concentrations of ionised $Ca^{2+}$ within the body depends on the integration of a number of specialised tissues (discussed previously in Chapters 4 and 5). Briefly, the intestinal CaR has a pivotal role in maintaining $Ca^{2+}$ homeostasis and is purported to modulate the synthesis/release/action of various hormone factors which (in addition to other tissue specific effects) are known to influence the trafficking of $Ca^{2+}$ across the intestine. For example, transition of the vitamin D endocrine system in the euryhaline rainbow trout (*Oncorhynchus mykiss*), from promoting intestinal absorption of $Ca^{2+}$ to its inhibition, and *vice versa*, is thought to be under the control of a CaR. The receptor is considered to regulate expression of vitamin D receptors in the intestine in response to changes in environmental salinity, and specifically the external $Ca^{2+}$ concentration (Larsson *et al.*, 2003). This would be consistent with previous work in rat parathyroid cells where it has been demonstrated that extracellular $Ca^{2+}$ regulates vitamin D receptor mRNA expression (Garfia *et al.*, 2002) *via* the CaR (Rodriguez *et al.*, 2007).

Parathyroid hormone-related protein (PTHrP) is a hyper-calcaemic factor in teleost fish, likely produced by the pituitary gland (Fraser *et al.*, 1991; Danks *et al.*, 1993), and has been shown to significantly increase intestinal $Ca^{2+}$ uptake by the gills and intestine (Guerreiro *et al.*, 2007). The caudal neurosecretory system (CNSS) consists of a collection of large neurosecretory cells, called Dahlgren cells, localised in the posterior region of the spinal cord. These cells are thought to be involved in a range of physiological processes including osmoregulation, reproduction and nutrition (Winter *et al.*, 2000). Co-localisation of PTHrP and the CaR to Dahlgren cells from the European flounder suggest that the synthesis and/or secretion of PTHrP may also be under the control of $Ca^{2+}$ signals from the nervous system (Ingleton *et al.*, 2002). Similarly, stanniocalcin is one of the principal hypo-calcaemic factors in marine teleosts which has direct effects on intestinal $Ca^{2+}$ transport, and is also

mediated by a CaR responding to changes in circulating levels of $Ca^{2+}$ (Radman *et al.*, 2002).

**2.2 Salinity sensor**

The broad distribution of the CaR in tissues that do not necessarily have an intrinsic role in $Ca^{2+}$ homeostasis indicates a much broader relevance for the receptor. For example, with modulation of CaR sensitivity by ionic strength (Quinn *et al.*, 1998), this has led to the consideration that the receptor also operates as a salinity sensor, capable of detecting alterations in the salinity of the surrounding water (Nearing *et al.*, 2002). Molecular studies detailing the cloning and characterisation of the teleost CaR have indeed confirmed that its activation is also modulated by changes in ionic strength (Nearing *et al.*, 2002; Loretz *et al.*, 2004).

The identification of a specific $Ca^{2+}$-sensing mechanism in the olfactory system of the euryhaline sea bream (*Sparus aurata*), where the firing rate of the olfactory nerve varied with environmental $Ca^{2+}$ pointed to a role for the CaR in the detection of external $Ca^{2+}$ concentrations (Hubbard *et al.*, 2000). Identification and localisation of a CaR to the olfactory nerves and epithelium of not only the sea bream (Flanagan *et al.*, 2002) but also the stenohaline freshwater goldfish, *Carassius auratus* (Hubbard *et al.*, 2002), as well as the anadromous Atlantic salmon, *Salmo salar* (Nearing *et al.*, 2002; Dukes *et al.*, 2006) and the euryhaline puffer fish, *Fugu rubripes* (Naito *et al.*, 1998). These observations would appear consistent with involvement of the receptor in communication, *via* the central nervous system, of changes in environmental $Ca^{2+}$ leading to either behavioural avoidance of, or physiological adjustment to, a salinity challenge.

It has been speculated that due to expression of the receptor in the olfactory epithelium along with the pituitary and CNSS, suggests that signals originating from changes to external salinity (and specifically $Ca^{2+}$), are integrated with endocrine and neuroendocrine activity (Loretz *et al.*, 2004; Loretz, 2008). The pituitary and CNSS are not only involved in $Ca^{2+}$ homeostasis but also osmoregulation. For example, many of the hormones that modulate osmoregulatory functions within the body, such as ion and fluid transport by the intestine, originate from the pituitary gland (Buddington and Krogdahl, 2004) including oxytocin (Baldisserotto and Mimura, 1997), arginine vasotocin (Bond *et al.*, 2002) and prolactin (Manzon, 2002). Similarly, neuroendocrine factors synthesised and secreted by

the CNSS, specifically urotensins I and II, are also considered to have a role in osmoregulatory adaptation (Winter *et al.*, 2000).

### 2.3 Intestinal ion and fluid transport

The CaR is expressed in the intestine of a number of teleost species including the sea bream (Flanagan *et al.*, 2002; Hang *et al.*, 2005), Atlantic salmon and winter flounder, *Pseudopleuronectes americanus* (Nearing *et al.*, 2002), tilapia, *Mossambicus oreochromis* (Loretz *et al.*, 2004) and European flounder (Cooper, C. A., personal communication). Aside from the peripheral influence of the CaR *via* the endocrine system, its presence along the gut itself implies a more direct role for the receptor. Apical localisation (Nearing *et al.*, 2002; Loretz *et al.*, 2007), places the CaR in direct contact with the contents of the gut where it will be able to monitor and respond to changes in extracellular $Ca^{2+}$. Along with the modulation of receptor sensitivity by ionic strength, which *in vivo* would confer a substantial modification to the $EC_{50}$ for $Ca^{2+}$ (discussed in Chapter 6, Section 2.4), makes the CaR an attractive candidate for the regulation of intestinal $HCO_3^-$ secretion. Generally, evidence of a role in ion and fluid transport along the gastrointestinal tract is limited to a handful of experiments which have shown that activation of the CaR in rat colonic crypts (by extracellular $Ca^{2+}$) reverses ion and fluid secretion and promotes absorption (Geibel *et al.*, 2006). A similar influence has been proposed for fluid transport in the gastric glands of the amphibian stomach (Gerbino *et al.*, 2007). In other types of transporting epithelia too the CaR has been shown to exert an influence on ion and fluid movement, for example, reducing water re-absorption in the kidney by altering the hydraulic permeability of the inner medullary collecting duct (Sands *et al.*, 1997). Similarly in rat pancreatic ducts a CaR monitors extracellular $Ca^{2+}$ moderating fluid secretion accordingly (Bruce *et al.*, 1999). More pertinently, fluid re-absorption by the urinary bladder of the winter flounder is modulated by a CaR which regulates NaCl co-transport (Nearing *et al.*, 2002).

### 2.4 Epithelial barrier function

The CaR has been localised within the intestinal tissue itself, in particular to isolated cells within the lamina propria of the sea bream, which were tentatively identified as leucocytes (Flanagan *et al.*, 2002). This indicates that the receptor could be poised to influence the

barrier function of the intestinal epithelia by acting as a sensor or messenger between the internal and external compartments. Described as the largest immunologic organ in the vertebrate body (Takahashi and Kiyono, 1999), the gut is continuously exposed to the environment where it is likely to encounter numerous pathogens; hence expression of the CaR in white blood cells, as observed by Flanagan *et al.* (2002), could imply a possible role in an immune response. Furthermore, activation of the CaR in colonic myofibroblast cells, found between the epithelia and lamina propria, leads to the synthesis and secretion of factors known to regulate cell proliferation (Peiris *et al.*, 2007). The intestine is in a constant state of renewal, and in mammals cell proliferation takes place in specialised structures known as the crypts of Lieberkühn (Sancho *et al.*, 2003). The activity of the CaR is also modulated by polyamines (Quinn *et al.*, 1997), which are crucial factors for normal rates of cell proliferation and differentiation required to sustain the rapid turnover of gastrointestinal epithelial cells (Loser *et al.*, 1999), hence, the involvement of a CaR in these processes has also been considered (Chattopadhyay *et al.*, 1998; Hebert *et al.*, 2004). Interestingly, the crypts of Lieberkühn have been noted as conspicuously absent from the teleost intestine (Field *et al.*, 1978). However, while the process of epithelial regeneration has not been extensively studied in fish, recent work has identified equivalent structures from the intestine of the common wolf-fish (*Anarhichas lupus*). These regularly distributed crypts reach into the lamina propria and were recognised as the site of cell re-generation (Hellberg and Bjerkas, 2005). There are also other reports of the CaR acting as a regulator of proliferation and differentiation in a number of other cell types including the human colon cell line, Caco-2 (Kallay *et al.*, 1997), ovarian surface epithelial cells (Hobson *et al.*, 2000), osteoblasts (Dvorak *et al.*, 2004), keratinocytes (Tu *et al.*, 2004) and mesangial cells of the glomerulus (Kwak *et al.*, 2005).

**2.5 Digestion and nutrient sensing**

The intestine is not only a major osmoregulatory organ in teleosts, but as well as contributing to $Ca^{2+}$ homeostasis it will naturally be responsible for digestion and nutrient absorption, and subsequently need to successfully integrate these essential functions. This is perhaps exemplified by lack of disturbance in the efficacy of protein digestion and amino acid absorption by the rainbow trout (Dabrowski *et al.*, 1986), and postprandial acid-base balance (i.e. the alkaline tide) in the flounder (Taylor *et al.*, 2007), maintained in either

freshwater or seawater. Similarly, there were no disturbances in systemic $Ca^{2+}$ homeostasis in Gulf toadfish (*Opsanus beta*) following consumption of a $Ca^{2+}$-rich meal (Taylor and Grosell, 2006a). However, while knowledge of the regulating mechanism(s) behind these capabilities remains little understood, there is potential scope for involvement of a CaR receptor in some or all of these processes.

In addition to its various cationic agonists, the CaR can also be activated by a large number of L-amino acids, showing a particular preference for those possessing aromatic groups. In addition, amino acids can also enhance the sensitivity of the CaR by reducing the $EC_{50}$ for receptor activation by $Ca^{2+}$ (Conigrave *et al.*, 2000). These findings suggest that the CaR offers an intriguing molecular link between protein digestion and $Ca^{2+}$ metabolism, as well as explanations for long-standing observations on the ability of amino acids to directly stimulate various phases of digestion, such as the secretion of gastric acid and release of pancreatic fluid (Conigrave *et al.*, 2000; Brown and MacLeod, 2001; Busque *et al.*, 2005; Conigrave and Brown, 2006). Such evidence for a similar mechanism of nutrient sensing in the teleost intestine is currently limited to structural similarities in the receptor, specifically the serine residues located in the extracellular domain of the CaR which have been linked to the binding of $Ca^{2+}$ and amino acids (Zhang *et al.*, 2002). These residues were found conserved (at identical positions) in the CaR cloned and characterised from the tilapia (Loretz *et al.*, 2004). Such consistencies in structure across taxa may explain the widespread expression of the receptor along the gastrointestinal tract in fish (Flanagan *et al.*, 2002; Nearing *et al.*, 2002; Loretz *et al.*, 2004), as well as mammals (Cima *et al.*, 1997; Gama *et al.*, 1997; Cheng *et al.*, 1999; Chattopadhyay *et al.*, 1998; Rutten *et al.*, 1999; Hebert *et al.*, 2004).

From a comparative perspective, a similar capacity of the teleost CaR to sense amino acids alongside $Ca^{2+}$, contributes an additional layer of intrigue to the prospective role(s) of the receptor in the intestine, specifically, integrating the complex demands from osmoregulation, $Ca^{2+}$ homeostasis and digestion. For example, these alternate modes of receptor operation may be beneficial considering that drinking rate (and consequently $Ca^{2+}$ intake) increases following ingestion of a meal in seawater-adapted flounder (Whittamore, J. M. and Wilson, R. W., unpublished observations), in addition to the $Ca^{2+}$ content of the meal itself. Furthermore, amino acids are recognised as potent stimulators of intestinal cholecystokinin (CCK) release in mammals (Konturek *et al.*, 1973; Douglas *et al.*, 1988),

with the CaR proposed as a potential mediator of this response (Conigrave and Brown, 2006). CCK is an important neuropeptide which has a number of vital roles in digestion including control of gall bladder motility, pancreatic enzyme secretion and the slowing down of gastric emptying (Crawley and Corwin, 1994). Interestingly, the release of intestinal CCK to the blood circulation has also been documented in response to amino acids within the lumen of the teleost intestine (Aldman and Holmgren, 1995; Koven *et al.*, 2002).

**2.6 Aims and objectives**

A brief review of the existing literature has revealed that the CaR possess a range of characteristics that make it ideally suited to operating within the intestine. However, it should be stressed that many of the functions of the CaR discussed for teleosts, particularly in relation to the gastrointestinal tract are speculative, based on a growing body of evidence that is primarily consigned to mammalian models and thus requires direct, physiologically relevant studies before any firm conclusions can be made for teleosts. The aim of the present study was therefore to evaluate whether the CaR does indeed have a role in ion and fluid transport by the teleost intestine, and in particular the regulation of $HCO_3^-$ secretion. The first set of experiments employed the *in vitro* paired gut sac technique to investigate whether altering the ionic strength or osmolality of the mucosal saline would modify the sensitivity of the receptor to luminal $Ca^{2+}$. This would mimic *in vivo* changes in gut fluid composition as it moves along the intestine and any enhancement of receptor sensitivity to $Ca^{2+}$ might reveal a response in $HCO_3^-$ secretion, which has so far eluded detection *in vitro*. While there are some compelling arguments supporting a role for the CaR in regulating $HCO_3^-$ secretion (Wilson *et al.*, 2002), as well as ion and fluid transport (discussed in Chapter 4, Section 5.3.4) by the teleost intestine, there is currently little direct evidence of a functional role for the receptor in these processes *in vitro*. A second set of gut sac experiments sought to address this by applying known agonists of the receptor in a bid to explore the possibility that the CaR is involved in the regulation of ion and fluid transport. Although the effects of elevated mucosal $Ca^{2+}$ using the *in vitro* gut sac preparation have proven ambiguous so far, there is however a clear, definitive response to $Ca^{2+}$ following perfusion of the intestine *in vivo* (Chapter 5). The final set of experiments involved perfusing the intestine *in vivo* with CaR agonists against a background of 10 mM $Ca^{2+}$ and

observing the effects on $HCO_3^-$ production and secretion as well as accompanying ion and fluid transport. If successful in providing some direct, functional evidence for a CaR, this study would open up opportunities to integrate the physiological responses at the tissue and whole animal level, with a detailed molecular characterisation of the intestinal CaR in a bid to more fully understand its role in a model euryhaline teleost.

## 3. Materials and Methods

### 3.1 Experimental animals

European flounder, *Platichthys flesus,* were obtained from local fishermen in Flookburgh, Cumbria, U.K. (n = 19, mean body mass 413 ±19 g and 31.4±0.4 cm, total length) and Fremington, North Devon, U.K. (n = 9, mean body mass 450 ±41 g and 33.7 ±0.7 cm, total length). A small number of fish used in these experiments had been purchased from Aquarium Technology, Dorset, UK (n = 4, mean body mass 335 ±29 g and 31.6 ±1.0 cm, total length). All fish were transported to the School of Biosciences, University of Exeter where they were held in marine aquarium facilities in 150 litre tanks of flowing, aerated artificial seawater as part of a recirculating seawater system maintained at 34.2 ±0.3 ppt and 11.6 ±0.3 °C, under a 12 hour light: dark photoperiod. At least 7 days were allowed for the fish to acclimate after arriving in the aquarium. Food was typically withheld for 72 hours prior to experimentation, otherwise the fish were maintained on a diet of fresh ragworm (*Nereis virens*) fed once per week.

### 3.2 *In vitro* experiments

#### 3.2.1 General experimental approach

The design, preparation and execution of the following experiments were identical to the paired gut sac protocol described previously (Chapter 4, Section 3.3). Briefly, gut sacs were made from the anterior, mid and posterior thirds of the intestine and experiments involved an initial 2 hour incubation period with a 'control' mucosal saline containing 5 mM $Ca^{2+}$, before rinsing the sac and refilling with a high $Ca^{2+}$ (20 mM $Ca^{2+}$) mucosal saline and incubating for a further 2 hours.

### 3.2.2 Is $HCO_3^-$ secretion modulated by ionic strength and osmolality?

Having already demonstrated that $HCO_3^-$ secretion is specifically stimulated by $Ca^{2+}$ in the flounder intestine using the *in vitro* Ussing chamber approach with a reduced osmolality saline (Wilson *et al*., 2002), it was logical that this same question, asked previously for the killifish (Chapter 6, Section 2.4), was also considered using flounder gut sacs. The first set of experiments in this study therefore employed a reduced ionic strength mucosal saline, using mannitol to maintain osmotic pressure at ~320 mOsm $kg^{-1}$, identical to the 'regular' serosal saline used previously (see Chapter 4, Section 3.2). A second set of experiments subsequently evaluated the influence of the 'reduced osmolality' mucosal and serosal salines (~280 mOsm $kg^{-1}$) on the response of the tissue to elevated luminal $Ca^{2+}$. A detailed description of each of these salines is listed in Table 7.1 below.

### 3.2.3 Applying agonists of the calcium-sensing receptor (CaR)

Two commonly used agonists of the CaR, gadolinium ($Gd^{3+}$) as $GdCl_3$, and neomycin (in the form of neomycin sulfate) were applied (separately) in gut sacs as part of the following investigation. These experiments were carried out with the 'reduced ionic strength' mucosal saline as conditions of reduced ionic strength are recognised as increasing the sensitivity of the receptor to its agonists (Quinn *et al*., 1998; Nearing *et al*., 2002; Loretz *et al*., 2004), and would therefore present the most favourable conditions for activation of the receptor. With no studies having previously documented the use of these agonists in the intestine another consideration was the appropriate dosage at which to apply them. The CaR is more sensitive to changes in net charge rather than specific ligand. This explains why members of the trivalent lanthanide series, such as $Gd^{3+}$, are capable of activating the receptor with concentrations in the micro-molar range, as opposed to the typical milli-molar concentrations required for $Ca^{2+}$ or $Mg^{2+}$ (McLarnon and Riccardi, 2002). Similarly, the potency of aminoglycoside antibiotics (e.g. neomycin, kanamycin and gentamicin) correlates with the number of attached amino groups (McLarnon *et al*., 2002). In trials, neomycin was found to be the most potent of these, possessing 6 amino groups and thus having a potential maximum charge of 6+ (Josepovitz *et al*., 1982; McLarnon *et al*., 2002). Both $Gd^{3+}$ and neomycin have been used successfully by numerous investigators, but appear largely confined to studies on individual cells or cultured cell lines. Perhaps the

most pertinent use of these agonists, in relation to the present study, has been with bladder 'sacs', made from the urinary bladder of the winter flounder, where they were applied at concentrations ranging from 19-250 µM for $Gd^{3+}$ and 50-250 µM for neomycin (Nearing *et al*., 2002). For the current experiments it was eventualy decided to use a relatively high concentration of each compound (1 mM) dissolved directly in the 'control' mucosal saline. For each of these experiments a total of 3 successive 2 hour incubations periods was undertaken as opposed to two. The first two incubations consisted of the 'control' saline followed by 'control + 1 mM CaR agonist'. A third flux period was introduced to observe whether the effects on ion and fluid transport (if any) were reversible following

Table 7.1: The inorganic salts and additional solutes used in the composition of the mucosal and serosal salines employed in the following experiments. The concentration of each component is given in mmol $l^{-1}$. Osmolarity was calculated from the osmotic coefficient of each constituent and presented in mOsm$l^{-1}$.

| | **Mucosal salines** | | | | **Serosal salines** | |
|---|---|---|---|---|---|---|
| | **Control** | | **High $Ca^{2+}$** | | | |
| | **Reduced ionic strength**** | **Reduced osmolality** | **Reduced ionic strength** | **Reduced osmolality** | **Regular** | **Reduced osmolality** |
| NaCl | 49.0 | 45.0 | 49.0 | 45.0 | 146.0 | 130.0 |
| KCl | 5.0 | 5.0 | 5.0 | 5.0 | 3.0 | 2.5 |
| $MgCl_2.6H_2O$ | 25.0 | 32.5 | 10.0 | 17.5 | - | - |
| $CaCl_2.6H_2O$ | 5.0 | 5.0 | 20.0 | 20.0 | 2.0 | 2.0 |
| $MgSO_4.7H_2O$ | 70.0 | 80.0 | 70.0 | 80.0 | 0.9 | 0.9 |
| $NaHCO_3$ | 1.0 | 1.0 | 1.0 | 1.0 | 8.0 | 8.0 |
| $Na_2HPO_4$ | - | - | - | - | 0.5 | 0.5 |
| $KH_2PO_4$ | - | - | - | - | 0.5 | 0.5 |
| HEPES (Free acid) | - | - | - | - | 4.0 | 4.0 |
| HEPES ($Na^+$ salt) | - | - | - | - | 4.0 | 4.0 |
| D-Mannitol | 58.0 | - | 58.0 | - | - | - |
| D-Glucose | - | - | - | - | 6.0 | 6.0 |
| L-Glutamine | - | - | - | - | 6.0 | 6.0 |
| L-Glutathione | - | - | - | - | 1.0 | 1.0 |
| pH | - | - | - | - | 7.80 | 7.80 |
| Osmolarity* | 321 | 284 | 320 | 283 | 323 | 292 |

*These are the theoretical, calculated osmolalities of the salines as originally made up. The osmotic pressure of the mucosal, and corresponding serosal saline, were matched exactly prior to each experiment.

**It should be noted that prior checks of saline pH revealed that both neomycin sulphate and $GdCl_3$ were slightly acidic and it was therefore necessary to correct for this using NaOH.

application of either $Gd^{3+}$ or neomycin, as would be expected following activation of the CaR. Indeed, Nearing *et al.* (2002) found that the inhibition of $J_v$ in bladder sacs after administration of either $Gd^{3+}$ of neomycin was fully reversible. Thus, at the end of the second incubation period the sac was emptied and gently rinsed 3 times with 1-1.5 ml of 'control' mucosal saline before being refilled and incubated for a further 2 hours.

### 3.2.4 Saline design and composition

A similar composition of reduced ionic strength and osmolality salines detailed in the previous chapter (Chapter 6, Section 3.6) was applied in the present work with the flounder and details are shown in Table 7.1. For the high $Ca^{2+}$ version of each mucosal saline the concentration of $Ca^{2+}$ was increased four-fold from 5 to 20 mM in keeping with previous *in vitro* experiments (Chapters 4 and 6).

## 3.3 *In vivo* experiments

### 3.3.1 Experimental approach and salines

The surgical procedures, perfusion protocol along with the sampling regime and analysis detailed in Chapter 5 (Section 3.2) were followed for the present *in vivo* experiments. Once surgery was complete the intestine was perfused with saline containing either $Gd^{3+}$ or neomycin, the same agonists applied *in vitro*, with a nominal concentration of 1 mM chosen for each (Table 7.2).

## 3.4 Data presentation and analysis

Data are presented as mean ±SE. For the *in vitro* experiments, positive numbers were indicative of net absorption and negative numbers of net secretion. Differences in rates of ion and fluid transport between anterior, mid and posterior sections of the intestine were assessed by a two-way ANOVA, using a General Linear Modelling (GLM) procedure, where section of the intestine, and incubation period were factors. This analysis also included calculation of an interaction term between these factors. Differences in net fluxes between treatments were assessed by paired t-tests. Where appropriate, following multiple paired comparisons, a modified Bonferroni correction of the probability value

Table 7.2: The inorganic salts used in the composition of the *in vivo* perfusion salines employed by the present study. The concentration of each component salt is given in mmol $l^{-1}$. Theoretical osmolarity (mOsm $l^{-1}$) was calculated based on the osmotic coeficient of each salt.

| Salt | Perfusion treatment | | |
|---|---|---|---|
| | Control | Control (+ $Gd^{3+}$)* | Control (+ Neomycin) |
| NaCl | 50.0 | 50.0 | 50.0 |
| KCl | 5.0 | 5.0 | 5.0 |
| $MgCl_2.6H_2O$ | 80.0 | 80.0 | 80.0 |
| $CaCl_2.6H_2O$ | 10.0 | 10.0 | 10.0 |
| $MgSO_4.7H_2O$ | 10.0 | 10.0 | 10.0 |
| $GdCl_3$ | - | 1.0 | - |
| Neomycin sulfate | - | - | 1.0 |

*As before, the pH of the saline was corrected with NaOH since $GdCl_3$ is slightly acidic. However, unlike the acidic effect of neomycin observed with the reduced ionic strength mucosal saline (which contained mannitol), addition to the *in vivo* perfusion saline produced no change in pH.

associated with each additional paired t-test was applied to reduce the possibility of incurring a type-1 error (Holm, 1979; Sokal and Rohlf, 1994).

The data collected from the *in vivo* experiments with $Gd^{3+}$ and neomycin were compared to the 'control' perfusion saline. All data expressed as a proportion were arcsine transformed prior to analysis and significant differences between treatments tested for by one-way ANOVA using a General Linear Modelling (GLM) procedure. Post-hoc, pair-wise comparisons were made using Bonferroni simultaneous tests. For data failing to meet the assumptions of approximate normality and equality of variance, the non-parametric Kruskal-Wallis test was performed with post-hoc comparisons made using Dunns procedure. All statistical tests were accepted as significant at $P < 0.05$ following prior examination of approximate normality and equality of variance. Statistical analysis was carried out using Minitab v13.1 and graphs drawn using SigmaPlot v9.0.

## 4. Results

### 4.1 The influence of saline composition on $HCO_3^-$ secretion

#### 4.1.1 Regular mucosal saline

For the benefit of the reader and ease of comparison with the data from the 'reduced ionic strength' and 'reduced osmolality' salines used in the present study, data that had previously been collected using the 'regular' serosal saline and presented in Chapter 4 (Section 4.1) are shown again here in Figure 7.1.

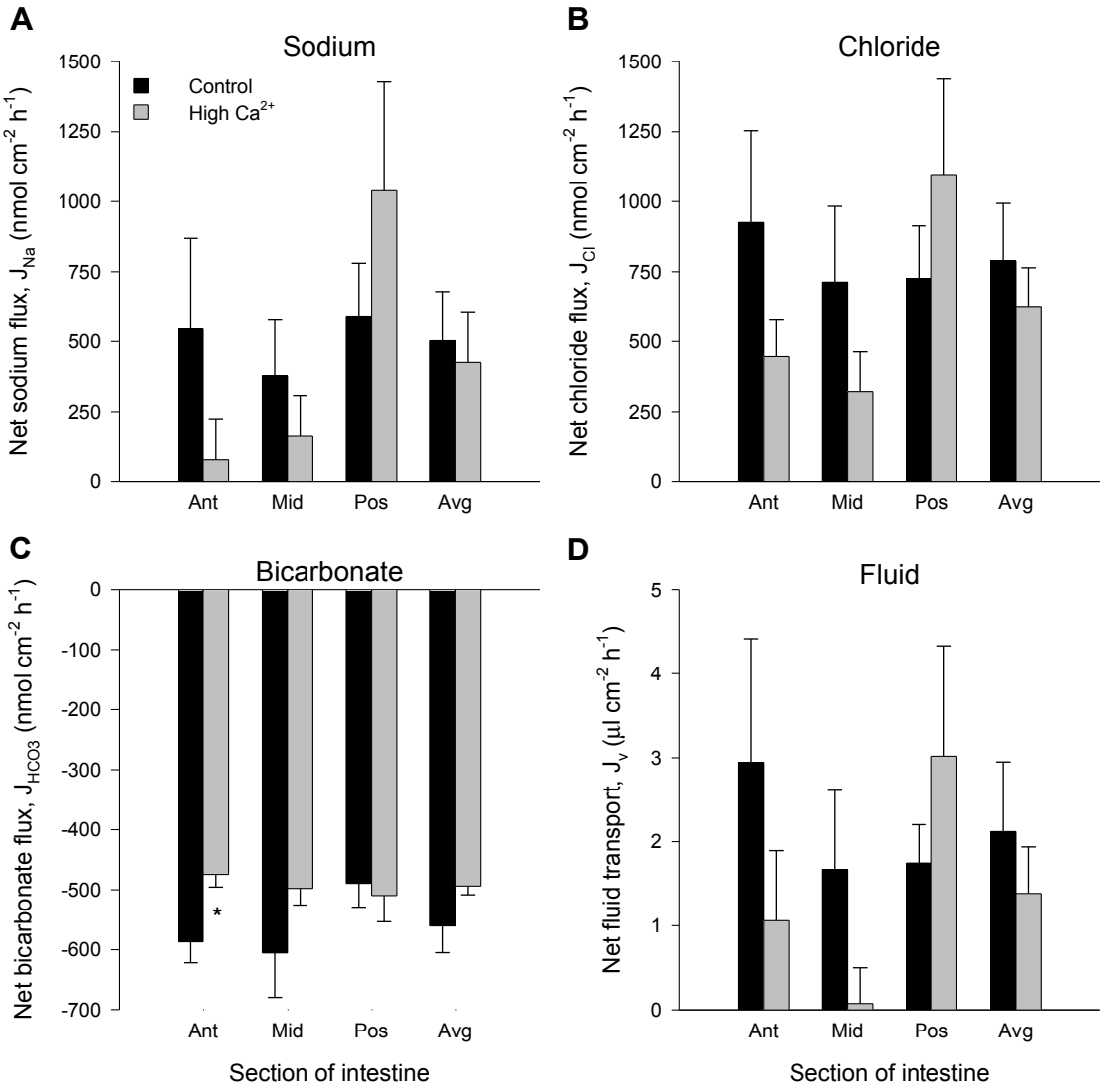



Figure 7.1: The mean (±SE) net fluxes of sodium, chloride and bicarbonate (nmol $cm^{-2}$ $h^{-1}$), alongside net fluid transport (µl $cm^{-2}$ $h^{-1}$) by gut sacs from the flounder intestine under control conditions (dark shading), and in response to a 15 mM increase in mucosal $Ca^{2+}$ concentration (light shading) using the 'regular' mucosal saline. These data were previously shown in Chapter 3 (Section 4.1).

### 4.1.2 Reduced ionic strength mucosal saline

There was a significant overall reduction in $HCO_3^-$ secretion by 12 % following the increase in mucosal $Ca^{2+}$ up to 20 mM, but secretion remained unchanged in the posterior section of the intestine. Even though there was a net secretion of $Na^+$ and very low rates

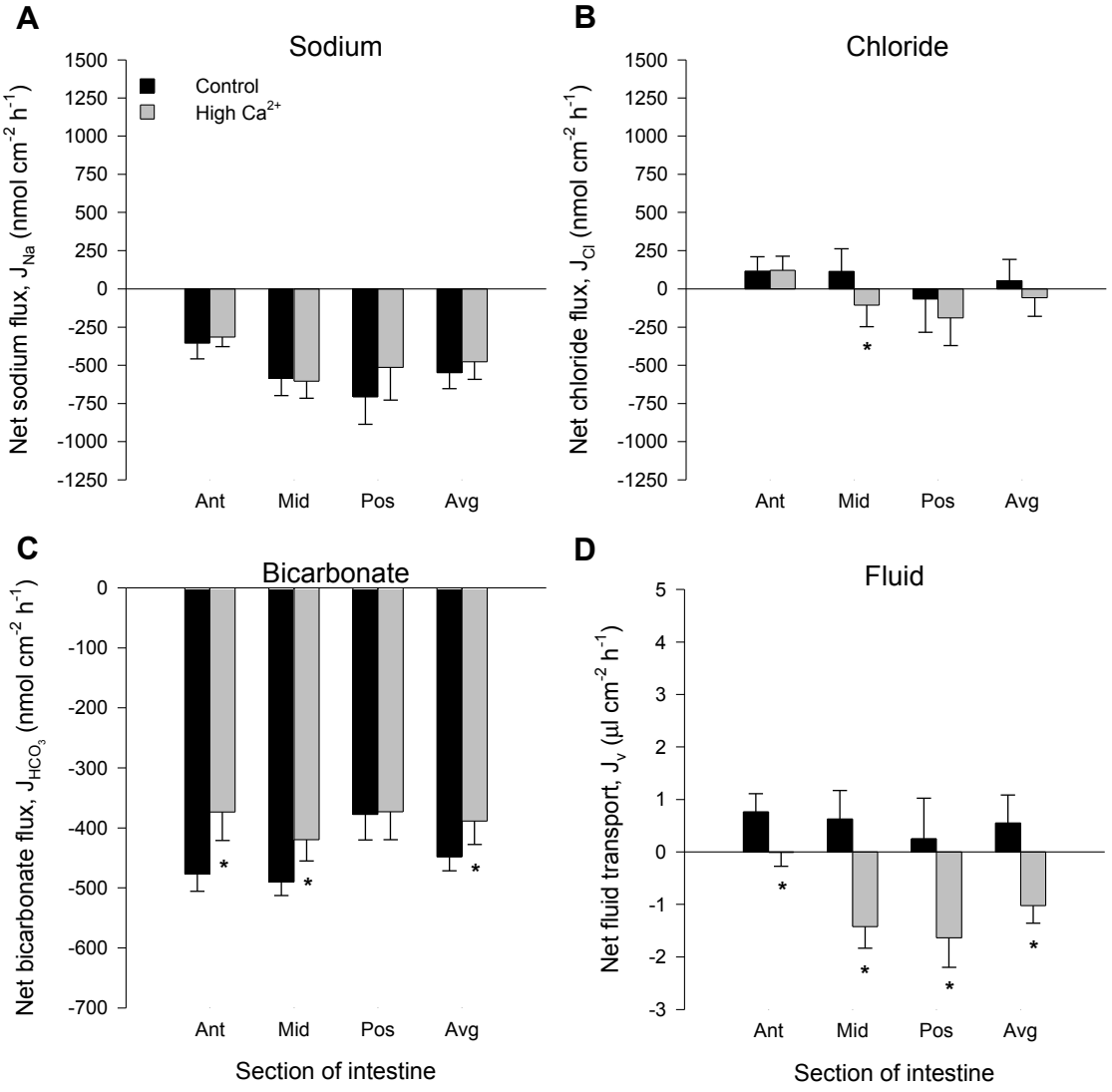



Figure 7.2: The mean (±SE) net fluxes of sodium, chloride and bicarbonate (nmol $cm^{-2}$ $h^{-1}$), alongside net fluid transport (µl $cm^{-2}$ $h^{-1}$) by gut sacs from the flounder intestine under control conditions (dark shading), and in response to a 15 mM increase in mucosal $Ca^{2+}$-concentration (light shading) using the 'reduced ionic strength' mucosal saline. Data are presented for anterior, mid and posterior sections, as well as the average value for the entire

intestine. Asterisks indicate a significant difference ($P < 0.05$) from corresponding the control incubation ($n = 6$).

of $Cl^-$ absorption, there was, on average, net fluid absorption from mucosa to serosa during the control incubation. However, under high $Ca^{2+}$ conditions, while net NaCl transport remained largely unchanged there was a significant turnaround of fluid transport rates, from absorption to secretion (Figure 7.2).

### 4.1.3 Reduced osmolality saline

With the osmotic pressure of the saline reduced by approximately 40 mOsm $kg^{-1}$ a very similar pattern of ion and fluid transport was observed (Figure 7.3) compared to the previous experiment using reduced ionic strength saline. Net $HCO_3^-$ secretion by both anterior and mid sections of the intestine was significantly reduced by 19 % and 13 %, respectively, and almost significant overall ($P = 0.054$). With comparable rates of $Na^+$ and $Cl^-$ transport shown previously in Figure 7.2, there was however a significant reduction in net $Na^+$ secretion for the mid and posterior portions of the gut. Fluid transport was again reversed in the presence of elevated mucosal $Ca^{2+}$, which was almost significant for the posterior gut sacs ($P = 0.064$).

## 4.2 The effect of calcium-sensing receptor (CaR) agonists on ion and fluid transport *in vitro*

### 4.2.1 Gadolinium ($Gd^{3+}$)

Applying 1 mM $Gd^{3+}$ resulted in a significant reduction in $HCO_3^-$ secretion for the anterior and mid sections which was not reversible. For the posterior gut sacs there was a

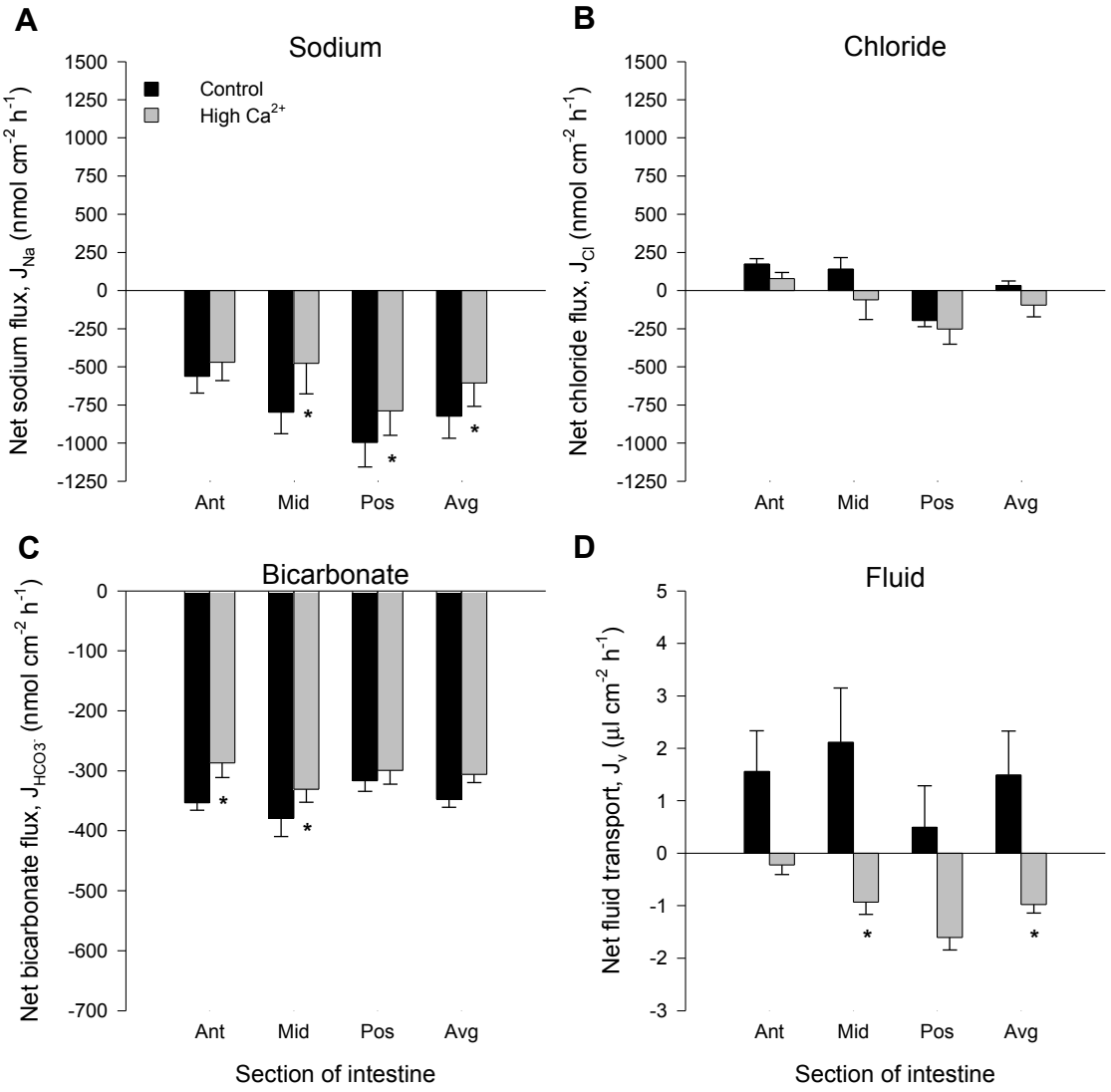



Figure 7.3: The mean (±SE) net fluxes of sodium, chloride and bicarbonate (nmol $cm^{-2}$ $h^{-1}$), alongside net fluid transport (µl $cm^{-2}$ $h^{-1}$) by gut sacs from the flounder intestine under control conditions (dark shading), and in response to a 15 mM increase in mucosal $Ca^{2+}$-concentration (light shading) using the 'reduced osmolality' mucosal saline. Data are presented for anterior, mid and posterior sections, as well as the average value for the entire intestine. Asterisks indicate a significant difference ($P < 0.05$) from corresponding the control incubation ($n = 6$).

non-significant 18 % reduction in net $HCO_3^-$ secretion rate but this was restored after rinsing out the $Gd^{3+}$ (Figure 7.4C). Indeed, the posterior portion of the intestine behaved a

little differently than more distal sections for the other ions too. While $Na^+$ and $Cl^-$ transport rates appeared largely unaffected there was a substantial, but not statistically significant increase in transport rates during the final incubation period (recovery after $Gd^{3+}$) compared to the initial control (Figures 7.4A and B). Interestingly, the response of fluid transport across treatments seemed to closely mirror that of $HCO_3^-$ secretion (Figure 7.4D).

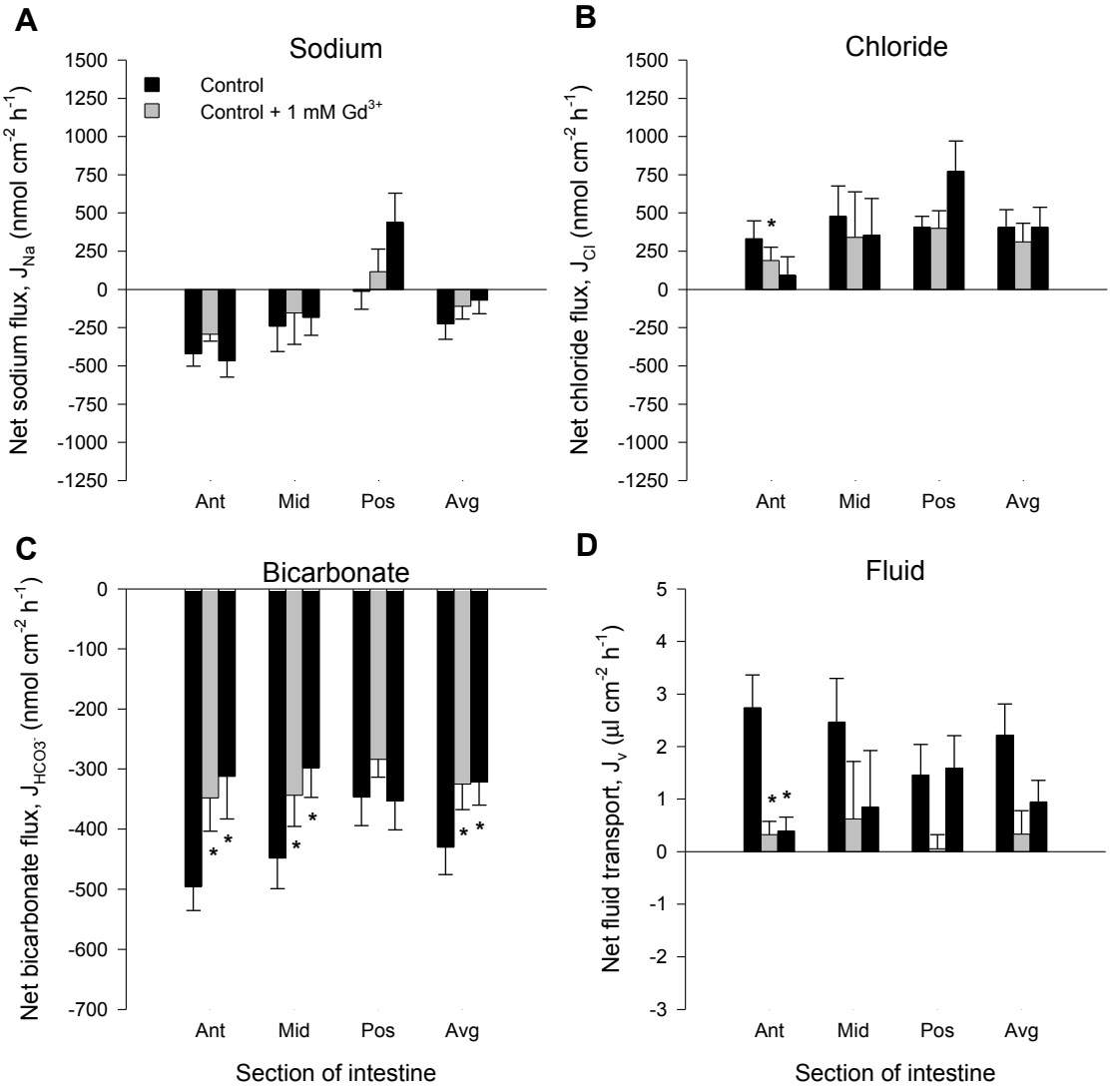



Figure 7.4: The mean (±SE) net fluxes of sodium, chloride and bicarbonate (nmol $cm^{-2}$ $h^{-1}$), alongside net fluid transport (µl $cm^{-2}$ $h^{-1}$) by gut sacs from the flounder intestine under control saline conditions (dark shading), and in response to 1 mM $Gd^{3+}$ (light shading) using the 'reduced ionic strength' mucosal saline. Data are presented for anterior, mid and

posterior sections, as well as the average value for the entire intestine. Asterisks indicate a significant difference ($P < 0.05$) from the corresponding initial control incubation ($n = 6$).

### 4.2.2 Neomycin

The response of ion and fluid transport to 1 mM neomycin displayed trends similar to those seen with $Gd^{3+}$ but were less convincing with no statistically significant differences between treatments (Figure 7.5).

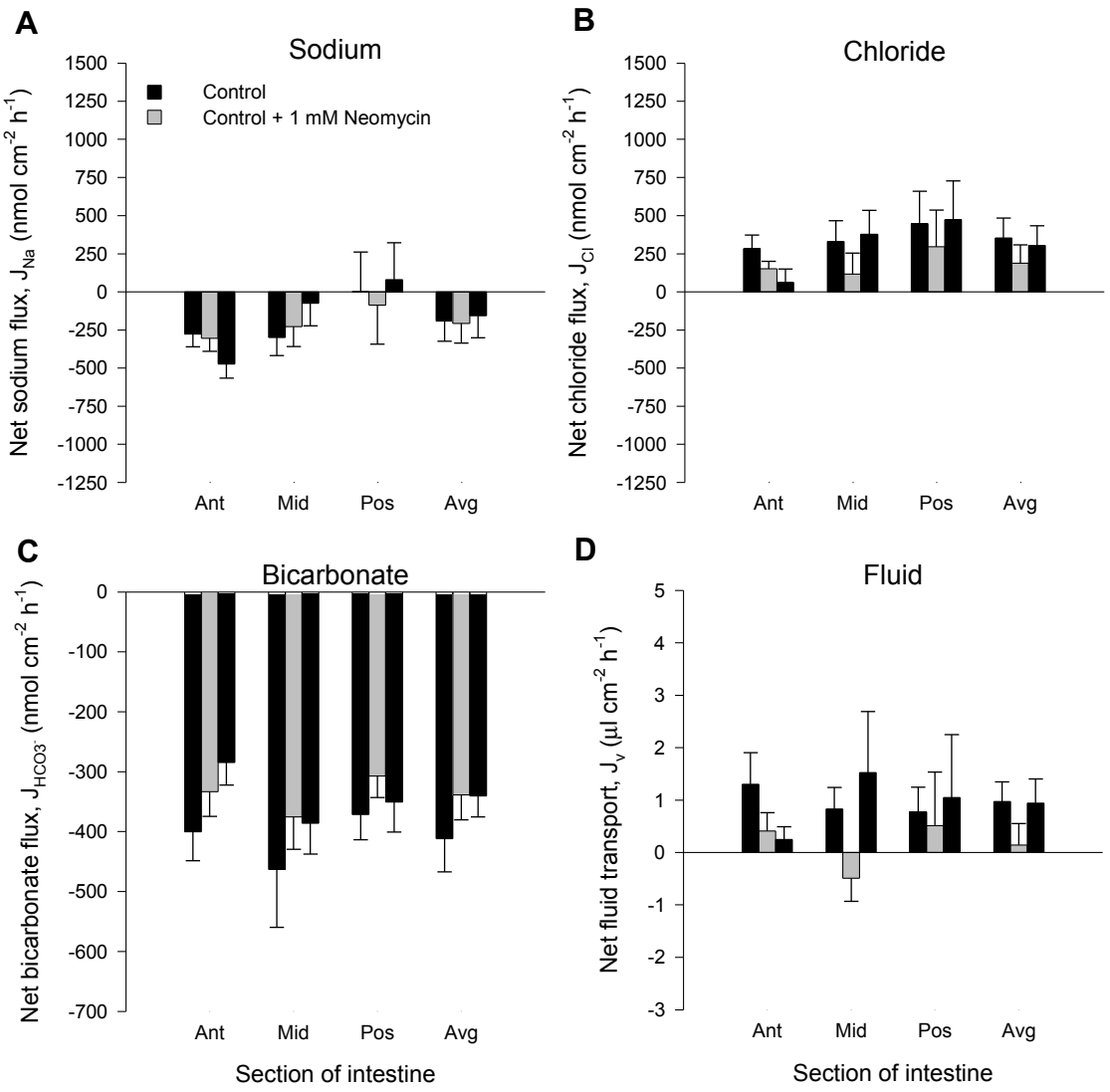



Figure 7.5: The mean (±SE) net fluxes of sodium, chloride and bicarbonate (nmol cm$^{-2}$ h$^{-1}$), alongside net fluid transport (µl cm$^{-2}$ h$^{-1}$) by gut sacs from the flounder intestine under

control conditions (dark shading), and in response to 1 mM neomycin (light shading) using the 'reduced ionic strength' mucosal saline. Data are presented for anterior, mid and posterior sections, as well as the average value for the entire intestine. Asterisks indicate a significant difference ($P < 0.05$) from the corresponding initial control incubation ($n = 6$).

## 4.3 The effect of calcium-sensing receptor (CaR) agonists on ion and fluid transport *in vivo*

### 4.3.1 Bicarbonate production and excretion

The inclusion of the CaR agonists $Gd^{3+}$ or neomycin did not enhance the rates of $HCO_3^-$ secretion or carbonate precipitation compared to controls (Figure 7.6).

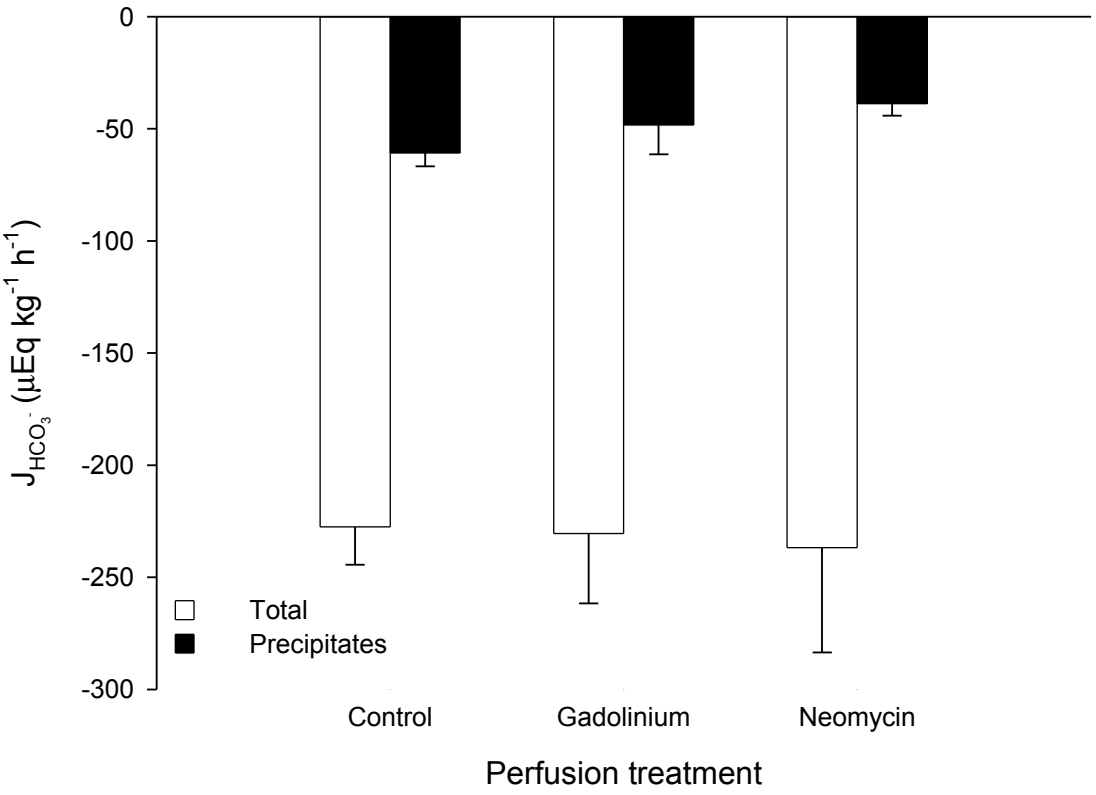



Figure 7.6: The mean (±SE) net production and excretion of bicarbonate ($HCO_3^- + 2CO_3^{2-}$) equivalents (µEq kg$^{-1}$ h$^{-1}$) by the intestine of the flounder perfused with salines containing 1 mM of the CaR agonists $Gd^{3+}$ or neomycin compared with the control over a total of 3 days. The open bars represent the total amount of bicarbonate equivalents (intestinal fluid +

rectal fluid + precipitates) produced and shaded barsshow the amount that had been incorporated into precipitates only (n = 8, 4 and 4 for the control, $Gd^{3+}$ and neomycin treatments, respectively

Similarly, the acid-base characteristics of the voided rectal fluid did not change significantly in the presenceof these agonists (Table 7.3).

Table 7.3: The mean (±SE) values of pH, $TCO_2$ (mM) and calculated $HCO_3^-$ equivalents (µ Eq $l^{-1}$) measured in rectal fluid samples from the flounder following perfusion of the intestine for 3 days with salines containing the CaR agonists, $Gd^{3+}$ and neomycin compared with the control (n = 8, 4 and 4 for the control, $Gd^{3+}$ and neomycin, respectively).

| | **Perfusion treatment** | | |
|---|---|---|---|
| | **Control** | **Gadolinium** | **Neomycin** |
| pH | 8.57 ±0.03 | 8.54 ±0.05 | 8.67 ±0.04 |
| $TCO_2$ (mM) | 61.2 ±4.7 | 50.1 ±8.6 | 57.8 ±4.5 |
| $[HCO_3^- + 2CO_3^{2-}]$ (µEq $l^{-1}$) | 68.4 ±5.6 | 55.9 ±10.0 | 65.8 ±4.7 |

### 4.3.2 Fluid transport

Figure 7.7 shows that over 3 days the proportion of fluid absorbed by the intestine was slightly reduced for fish undergoing perfusion with saline containing $Gd^{3+}$ or neomycin. Interestingly, between the control and neomycintreatments water absorption fell by 14 % (from 47.2 % to 33.3 %) but was not quite significantly different ($F_{2, 13} = 3.68$, P = 0.054).

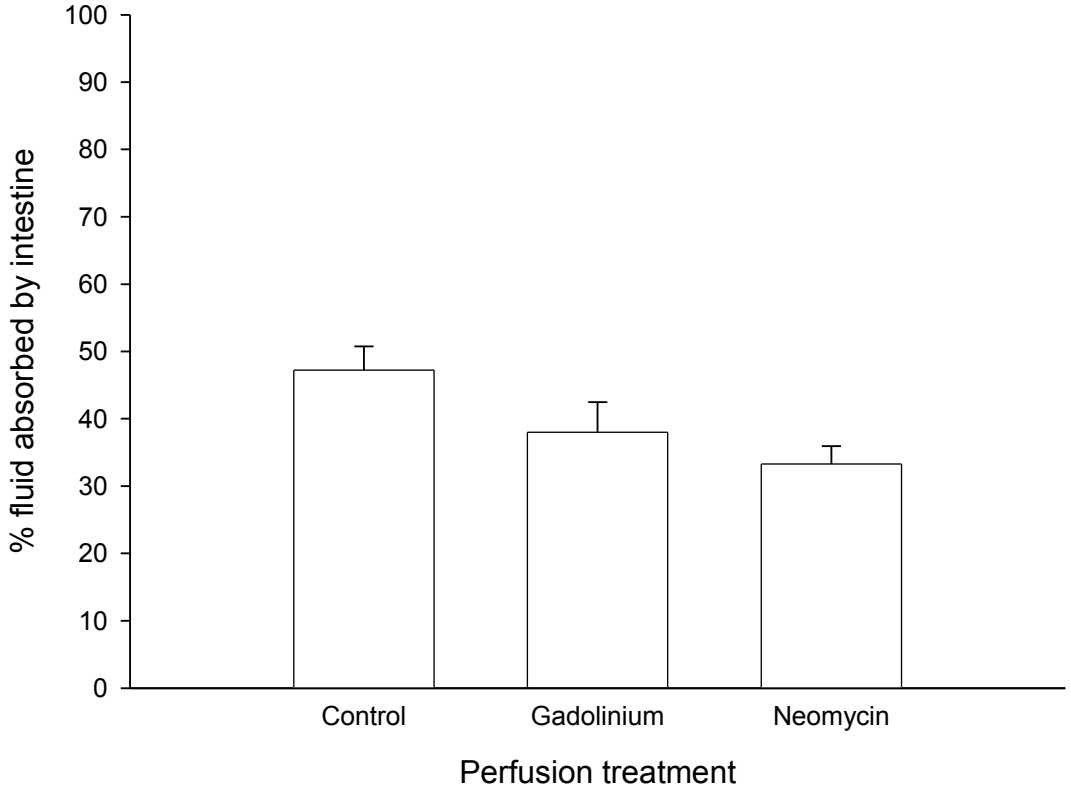



Figure 7.7: The mean (±SE) proportion of fluid absorbed by the flounder intestine following perfusion with salines containing 1 mM of the CaR agonists $Gd^{3+}$ or neomycin, compared with the control (n = 8, 4 and 4 for the control, $Gd^{3+}$ and neomycin treatments, respectively).

## 5. Discussion

The present study set out to demonstrate a role for the CaR in the marine teleost intestine, specifically its influence on $HCO_3^-$ secretion, and associated ion and fluid transport, both *in vitro* and *in vivo*. The first set of experiments investigated whether the response of the proposed receptor to $Ca^{2+}$ could be modulated by changes in ionic strength and osmolality of the mucosal saline *in vitro*. Demonstration of consistent ion and fluid transport rates over consecutive incubation periods, as described in Chapter 3, but in the presence of these

particular salines are missing from the data set. With additional time a series of consecutive control experiments would have been performed to help validate the data presented here. However, Grosell *et al*. (2005) did not find any significant differences in ion and fluid transport rates over 3 consecutive 2 hour incubations using a mucosal saline of a similar composition to the reduced ionic strength and reduced osmolality salines employed here. With a reasonable level of confidence it was assumed that flux rates would have been stable over consecutive incubation periods, but where relevant in the following discussion, this should be kept in mind as a potential caveat.

### 5.1 Why is $HCO_3^-$ secretion in gut sacs NOT stimulated by $Ca^{2+}$?

Altering the ionic strength and osmolality of the mucosal saline did not change the response of $HCO_3^-$ secretion to high $Ca^{2+}$ originally observed with the 'regular' saline (Figure 7.1), with the exception that the reductions in $HCO_3^-$ in the anterior and mid sections were now statistically significant (Figures 7.2 and 7.3). In terms of a CaR, these results are perhaps not too surprising given that the $EC_{50}$ for receptor activation by $Ca^{2+}$ would have only been reduced from around 5 to 2.5 mM in relation to the change in ionic strength during these experiments, based on the CaR characterised from dogfish kidney (Nearing *et al*., 2002). Since the concentration of $Ca^{2+}$ was increased by such a large amount (5 to 20 mM), it can be argued that the potential gain in sensitivity from reducing ionic strength in these experiments would have been largely irrelevant.

In spite of this, and the success of *in vivo* experiments exploring the regulation of $HCO_3^-$ secretion by $Ca^{2+}$ (Wilson *et al*., 2002; Wilson and Grosell, 2003; Chapter 5), along with a clear demonstration of a role for $Ca^{2+}$-specific $HCO_3^-$ secretion *in vitro* (Wilson *et al*., 2002), the gut sac experiments performed here have not matched this expectation in support of a role for $Ca^{2+}$ in the stimulation of $HCO_3^-$ secretion. While a substantial part of the discussion from Chapter 4 (Section 5.1) was devoted to demonstrating that there did not appear to be any obvious limitations to $HCO_3^-$ secretion it was decided to return to this possibility. The following discussion has sought to employ a more rigorous examination of the role for $Cl^-/HCO_3^-$ exchange. Specifically, attention has been focussed on the electrochemical driving forces behind $HCO_3^-$ secretion, in a bid to try and understand why secretion by gut sac preparations from the present study, as well as Chapter 4, appear to be

limited in terms of their capacity for $HCO_3^-$ secretion and subsequently failed to show the expected response to $Ca^{2+}$.

### 5.1.1 Thermodynamic considerations for $HCO_3^-$ secretion *in vitro*

Once more rates of $HCO_3^-$ secretion *in vitro* in these experiments appeared very consistent between 400 and 600 nmol $cm^{-2}$ $h^{-1}$, displaying little variation, irrespective of the accompanying changes in $Na^+$ and $Cl^-$ transport (Figures 7.1 to 7.5, see also Chapter 3, Section 4.3). A reason for such consistency could be the favourable electrochemical gradient for the movement of $HCO_3^-$ across the tissue from the serosal to mucosal side. With 8 mM serosal $HCO_3^-$ and 1 mM mucosal $HCO_3^-$ at the start, calculation of the Nernst equilibrium potential (for details see Chapter 6, Section 5.4.2) reveals that the serosal to mucosal gradient for the movement of $HCO_3^-$ would only be abolished if the transepithelial potential difference (TEP) were equal to or greater than +51.0 mV (serosa positive). The actual TEP, measured directly by Grosell *et al.* (2005) for flounder gut sacs, under near-identical conditions to the present study, was around -16 mV confirming a substantial electrochemical gradient for the movement of $HCO_3^-$ into the mucosal saline in these preparations. This value for the TEP compares well with -11.1 to -13.8 mV (Wilson *et al.*, 2002), and -13.5 to -14.5 mV (Wilson and Grosell, 2003), obtained from anterior sections of the flounder intestine mounted in the Ussing chamber under asymmetrical conditions, as well as *in vivo* measurements (-13.7 to -19.7 mV) made along the intestinal tract of this same species in two-thirds (21 ppt.) seawater (Bury *et al.*, 2001).

While useful, this cursory analysis does not impart the relative contribution of (secondary) active $HCO_3^-$ secretion since the energy stored in this gradient will be capable of not only driving apical $Cl^-/HCO_3^-$ exchange (Grosell, 2006), but also conductive exit across the apical membrane (Dixon and Loretz, 1986), and paracellular movement across the tight junction (Grosell *et al.*, 2001). Following re-arrangement of the Nernst equation, passive transepithelial movement of $HCO_3^-$ into the mucosal saline would be thermodynamically feasible up to a mucosal concentration of 15.4 mM $HCO_3^-$. Interestingly, Grosell *et al.* (2005) made a very similar prediction (17 mM) based on experimental observations of the relationship between measured $HCO_3^-$ secretion rates and the ratio of $HCO_3^-$ concentrations on the mucosal and serosal sides of the tissue. However, for the present study, the average concentration of $HCO_3^-$ recovered in the mucosal saline at the end of an incubation under

control conditions with reduced ionic strength mucosal saline was 7.9 ±0.3 mM (n = 54) far below these predicted limits.

### 5.1.2 The driving forces for $Cl^-/HCO_3^-$ exchange

A more informative approach to assessing the limitations of $HCO_3^-$ secretion would be to consider the electrochemical driving forces which exist across the apical and basolateral membranes *in vitro*, and more specifically the energy for apical $Cl^-/HCO_3^-$ exchange. Similar analyses have been carried out by Grosell *et al.* (2001) and Grosell (2006), based on *in vivo* data and the associated Nernst equilibrium potentials for $Cl^-$ and $HCO_3^-$. It was calculated that for anion exchange to be thermodynamically feasible in the anterior intestine the intracellular $HCO_3^-$ concentration would need to greater than 10 mM, and even higher in more distal regions *in vivo* (as the $HCO_3^-$ content of the gut fluid increases and $Cl^-$ decreases). Currently, there are no available measurements of intracellular $HCO_3^-$ concentration in the teleost intestine although estimates based on the Henderson-Hasselbach equation from previous *in vitro* studies range from 0.6 mM in the goby (Dixon and Loretz, 1986) to 1.5 mM in the Pacific sanddab (Grosell *et al.*, 2001). For the present gut sac preparations the cytosolic $HCO_3^-$ concentration was similarly calculated using the Henderson-Hasselbach equation:

$$pH_i = pK_{app} + \log_{10} ([HCO_3^-]_i / \alpha \times PCO_2) \qquad (1)$$

where, $pH_i$ is the intracellular pH (assumed to be ~7.4), $pK_{app}$ is the dissociation constant of carbonic acid (6.13) from Boutilier *et al.* (1984), $\alpha$ the solubility coefficient of $CO_2$ (0.057 mM mmHg), and $PCO_2$ the partial pressure of $CO_2$ within the cell, which was calculated as 3.8 mmHg for 0.5 % $CO_2$ (760 mmHg × 0.005), assuming there were no limitations on $CO_2$ diffusion across the cell membrane. Using this information, and re-arranging equation 1 gives an intracellular concentration of $HCO_3^-$ of 4.0 mM for the European flounder *in vitro*.

### 5.1.3 The electrochemical potential ($\Delta\mu$)

The next step to determining the thermodynamic feasibility for $Cl^-/HCO_3^-$ exchange *in vitro* involved calculation of the electrochemical potential (in kJ $mol^{-1}$) for $Cl^-$ and $HCO_3^-$ across

the apical ($\Delta\mu^{apical}$) and basolateral ($\Delta\mu^{basolateral}$) membranes using the following equation from Loretz (1995):

$$\Delta\mu = RT \ln([X_i] / [X_o]) + zF\Delta E \qquad (2)$$

where, $[X_i]$ and $[X_o]$ are the concentrations (mM) of the given ion in the cytoplasm and bathing solution, respectively. $\Delta E$ is the potential difference (V) across the specified membrane. R, T, z and F have their usual meanings. The apical membrane potential difference ($\Delta E^{apical}$) was taken as -100 mV with reference to the cytoplasm, under asymmetrical conditions (Loretz, 1995), and with TEP at -16 mV (Grosell *et al.*, 2005) the potential difference across the basolateral membrane ($\Delta E^{basolateral}$) was calculated as -84 mV (cytosol negative) using the following expression:

$$TEP = \Delta E^{apical} - \Delta E^{basolateral} \qquad (3)$$

Data on ionic activities in the cytoplasm of the teleost intestine are rather limited although intracellular $Cl^-$ has been determined for the winter flounder (Duffey *et al.*, 1979; Smith *et al.*, 1980) using an ion-specific microelectrode reporting an activity of about 30 mM. Along with the intracellular $HCO_3^-$ concentration of 4 mM, calculated from equation 1, the resulting electrochemical potentials are presented below (Table 7.4).

Table 7.4: The electrochemical potentials, $\Delta\mu$ (kJ $mol^{-1}$) of $Cl^-$ and $HCO_3^-$ across the intestinal epithelia of the European flounder at the beginning and the end of gut sac experiments *in vitro*. The mucosal concentrations are the actual mean (±SE) values measured during the control incubation period from experiments with the reduced ionic strength mucosal saline (n= 54). Where $\Delta\mu$ is <0 transport will be thermodynamically feasible. The direction of ion movement along its electrochemical potential gradient is indicated in parentheses where m = mucosa, c = cytoplasm and s = serosa.

| **Time (h)** | **Ion** | **Concentration (mM)** | | | **Δμ (kJ $mol^{-1}$)** | | |
|---|---|---|---|---|---|---|---|
| | | Mucosal | Cytoplasm | Serosal | Apical | Basolateral | Transepithelial |
| 0 | $Cl^-$ | 103.1 ±0.1 | 30 | 153 | 6.73 (c→m) | -4.25 (c→s) | 2.48 (s→m) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | $HCO_3^-$ | 1.1 ±0.0 | 4 | 8 | 12.70 (c→m) | -6.46 (c→s) | 6.24 (s→m) |
| 2 | $Cl^-$ | 100.7 ±0.7 | 30 | 153 | 6.78 (c→m) | | 2.53 (s→m) |
| | $HCO_3^-$ | 7.9 ±0.3 | 4 | 8 | 8.04 (c→m) | | 1.57 (s→m) |

active $Cl^-/HCO_3^-$ exchange to occur across the apical membrane can subsequently be calculated as the sum of the electrochemical potentials for $Cl^-$ and $HCO_3^-$ presented in table 7.4:

$$\Delta\mu^{Cl-/\,HCO3-} = \Delta\mu^{Cl-} + -\Delta\mu^{HCO3-} \qquad (4)$$

Note that the sign for $\Delta\mu$ $HCO_3^-$ is negative indicating movement out of the cell in the opposite direction to $Cl^-$. The electrochemical potential for $Cl^-/HCO_3^-$ exchange at the beginning of the incubation period was -5.92 kJ $mol^{-1}$. This shows that the process of apical $Cl^-/HCO_3^-$ exchange would have been thermodynamically permissible, driven by energy contained in the large outward gradient for $HCO_3^-$ exit. After 2 hours the $\Delta\mu$ for $Cl^-/HCO_3^-$ exchange would have been reduced more than 5-fold to -1.14 kJ $mol^{1}$. The above calculations serve to demonstrate that the energy for $Cl^-/HCO_3^-$ exchange would be greatly diminished, and lends support to the notion that $HCO_3^-$ secretion was indeed becoming limited towards the end of a 2 hour flux period. Even though, based on these figures, it was still theoretically possible for $Cl^-/HCO_3^-$ exchange to take place, in realistic terms this would be an unlikely prospect, as discussed below.

### 5.1.4 Is diffusion of $CO_2$ into the epithelial cell limited?

One of the pivotal assumptions of the above analysis will be the calculation of intracellular $HCO_3^-$, since the thermodynamics of the system dictate that with higher concentrations of $HCO_3^-$ in the cell, the more energy there will be to drive apical $Cl^-/HCO_3^-$ exchange. As a small neutral molecule, it was naturally assumed that $CO_2$ would freely diffuse across the basolateral cell membrane. However, since the permeability of *Xenopus* oocytes to $CO_2$ can be increased by the presence of the aquaporin water channel, AQP1 (Nakhoul *et al.*, 1998) this suggests that for some membranes permeability to $CO_2$ may be intrinsically limited,

and for some epithelia such properties may in fact be a necessity. For example, parietal and chief cells of the gastric glands, which secrete acid and pepsin into the stomach do not benefit from the protection of the mucus-bicarbonate barrier. Instead, they are uniquely resistant to acidification by being almost completely impermeable to $H^+$, $NH_3$ and $CO_2$ (Boron *et al*., 1994; Waisbren *et al*., 1994). It has more recently been shown that the apical membrane of colonocytes from the guinea pig also possess a reduced permeability to $CO_2$, more than 200 times lower than red blood cells (Endeward and Gros, 2005).
While the potential for AQP1 to act as a channel for $CO_2$ has been the subject of debate (Fang *et al*., 2002; Verkman, 2002), molecular simulations conclude that AQP1 could act as a $CO_2$ transporting channel in epithelia with low intrinsic $CO_2$ permeability (Hub and de Groot, 2006; Wang *et al*., 2007). Interestingly, AQP1 is abundantly expressed in the intestine of the Japanese eel (Aoki *et al*., 2003), European eel (Martinez *et al*., 2005) and sea bream (Raldua *et al*., 2008), but is localised almost exclusively to the apical membrane, with the exception of a novel aquaglyceroporin from the intestine of the sea bream (Santos *et al*., 2004). Additional support for the limited diffusion of $CO_2$ across the basolateral membrane of the flounder intestine is evident from the gut sac experiments in Chapter 4, where the serosal saline was gassed with 2 % $CO_2$ instead of 0.5 %, but the resulting mucosal $HCO_3^-$ secretion remained unchanged (Section 4.2, Figure 7.3). If $CO_2$ were able to move freely into the cell then the intracellular concentration of $HCO_3^-$ would have been expected to be considerably higher in the presence of 2 % $CO_2$ and therefore capable of driving additional $Cl^-/HCO_3^-$ exchange in gut sacs as demonstrated by (Grosell *et al*., 2005), yet this was clearly not the case.

### 5.1.5 Additional barriers to $Cl^-/HCO_3^-$ exchange in gut sacs

While intracellular $PCO_2$, and subsequently intracellular $HCO_3^-$, may have been over-estimated in terms of serosal $CO_2$ entering the cell, conversely, $CO_2$ from cellular metabolism would contribute to $PCO_2$. The flounder is considered to rely largely on endogenous $CO_2$ to fuel $HCO_3^-$ secretion. However, *in vitro*, without an intact blood supply, oxidative metabolism and the functional capacity of the tissue are likely to be depressed (Lifson and Parsons, 1957; Whitlock and Wheeler, 1964; Ochsenfahrt, 1979; Foulkes, 1996; Wilson *et al*., 2002). In the absence of extracellular $CO_2$ (i.e. $HCO_3^-/CO_2$-free serosal saline) the flounder intestine has been shown to produce sufficient $HCO_3^-$ capable of

driving secretion *in vitro*, albeit at much reduced rates, almost 50 % lower than measured here (Chapter 4, Section 4.3). To create high intracellular $HCO_3^-$ required to overcome these thermodynamic constraints it has been suggested that linkage of the cytoplasmic carbonic anhydrase (CA) to the apical $Cl^-/HCO_3^-$ exchanger forming a so-called 'metabolon' (Srere, 1987) would dramatically increase the activity of the exchanger (Grosell, 2006). By linking these processes, the diffusional distance between the CA and the $Cl^-/HCO_3^-$ exchanger is reduced thus permitting the development of high, localised intracellular concentrations of $HCO_3^-$ which would enhance transport (Sterling *et al.*, 2001; 2002).

However, if cellular metabolism, and consequently $CO_2$ production, was being limited *in vitro* the significance of such a metabolon would not necessarily be realised. Furthermore, the static conditions of the mucosal saline within the gut sac, which were neither gassed nor stirred, would provide the opportunity for a large unstirred boundary layer to form. Also, since the diffusion of $HCO_3^-$ through gastrointestinal mucus is 11 times slower than through saline (Livingston *et al.*, 1995), $HCO_3^-$ would be likely to concentrate against the membrane and be counter-productive to any localised $HCO_3^-$ concentrations built up on the inner side of the membrane. Indeed this situation would most probably diminish the gradient for $HCO_3^-$ secretion much more rapidly than suggested from the electrochemical profiles of $Cl^-$ and $HCO_3^-$ in Table 7.4. For example, even with the possibility for low rates of endogenous $HCO_3^-$ production and limited diffusion of $CO_2$ into the cell from the serosal side, if intracellular $HCO_3^-$ were indeed as high as 4 mM *in vitro*, then with an average concentration in the bulk fluid of 7.9 mM, it would only require a concentration of 13.4 mM $HCO_3^-$ within the unstirred layer on the mucosal side to eliminate $Cl^-/HCO_3^-$ exchange. In addition, with only another 2 mM the Nernst equilibrium potential of 15.4 mM (calculated in Section 5.1.1), for all forms of $HCO_3^-$ secretion would be reached.

### 5.1.6 Summary

In answer to the original question of why $Ca^{2+}$ has proven unsuccessful in stimulating $HCO_3^-$ secretion *in vitro*, a problem which has consistently plagued this research, it would appear to be down to the choice of technique. By finally considering the thermodynamics of $HCO_3^-$ transport it would appear that by its very nature the gut sac preparation was simply not suited to measuring large increases in $HCO_3^-$ secretion. As the $HCO_3^-$

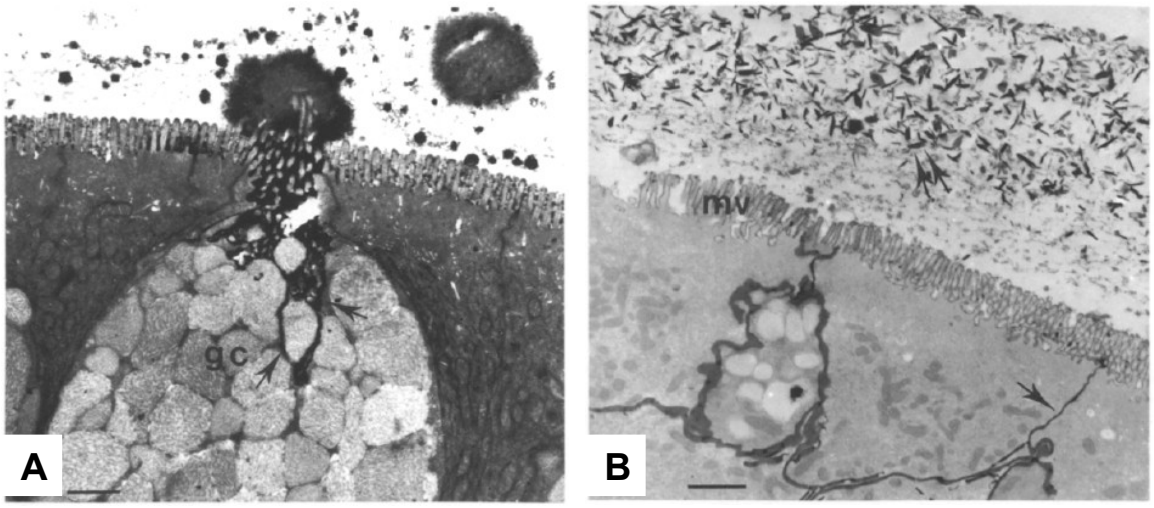
concentration in the enclosed, unstirred mucosal saline increased, the electrochemical gradient for secretion decreased (exemplified in Table 7.4). In contrast, the previous work of Wilson and co-workers (2002) employing the pH stat system where mucosal pH was clamped at 7.8, and the mucosal saline was well-stirred and oxygenated. Under these experimental conditions any such thermodynamic constraints would have been greatly minimised and the gradient for $HCO_3^-$ secretion maintained, subsequently producing a clear 47 % increase in the presence of elevated mucosal $Ca^{2+}$. While the ideas behind the use of the gut sac preparation to demonstrate anincrease in $HCO_3^-$ secretion were essentially sound, such experiments were likely to have been fundamentally flawed all along. If these arguments are correct then this would prove a bitterly disappointing conclusion to almost 3 years of time and effort in pursuit of using this technique to stimulate $HCO_3^-$ secretion *in vitro*.

### 5.2 The role of $CaCO_3$ production in $HCO_3^-$ secretion *in vivo*

The preceding sections have provided some illuminating discussion on the driving forces for $HCO_3^-$ which leads into the question of how secretion would be regulated *in vivo*. Concentrations of $HCO_3^-$ in the rectal fluid of the flounder range from 29 to as high as 114 mM, and with only 4 to 10 mM in the blood (personal observations), this is far beyond the equilibrium potential for $HCO_3^-$ exit. This point has been addressed by Grosell *et al.* (2001), and more recently in a review by Grosell (2006). It was suggested that linkage of the apical $Cl^-/HCO_3^-$ exchanger and CA (in the form of a metabolon), along with a high metabolic turnover of $CO_2$, producing a greater intracellular $PCO_2$, would together create the high, localised intracellular $HCO_3^-$ concentrations required to overcome these thermodynamic constraints *in vivo*. However, another aspect that was not considered by these investigators was the reduction in localised $HCO_3^-$ concentrations created by carbonate precipitation. As $CaCO_3$ crystallisation takes place against the cell surface (Humbert *et al.*, 1986; 1989) and hence will consume two molecules of $HCO_3^-$ in this region, then in combination with a high intracellular $HCO_3^-$ concentration, this could further enhance the gradient for $HCO_3^-$ to exit (Figure 7.8).

#### 5.2.1 Is a CaR required for $HCO_3^-$ secretion *in vivo*?

If the above hypothesis, illustrated in Figure 7.8, were correct then it would suggest that the rate of $HCO_3^-$ secretion *in vivo* would not necessarily be under the control of a CaR, instead a combination of physical factors could potentially regulate secretion *in vivo*, namely the ability to generate high intracellular $HCO_3^-$ concentrations (Grosell, 2006) and an enhanced gradient for $HCO_3^-$ exit created by $CaCO_3$ precipitation (Figure 7.8). This was previously considered by Wilson *et al.* (2002) who argued against a role for $CaCO_3$ in promoting the gradient for further secretion as the stimulation of $HCO_3^-$ secretion by $Ca^{2+}$ was equally apparent using the pH-stat system, where the gradient across the apical membrane was sustained by clamping luminal pH, $CO_2$ (and subsequently $HCO_3^-$) to a constant value. Contrary to this view, while this does indeed suggest a role for the CaR under such specific *in vitro* conditions, it is certainly does not seem to be the case *in vivo* and without prior understanding of how $Ca^{2+}$, and presumably the CaR, operates to increase $HCO_3^-$ secretion it is not possible to extrapolate this mechanism to the situation *in vivo*.



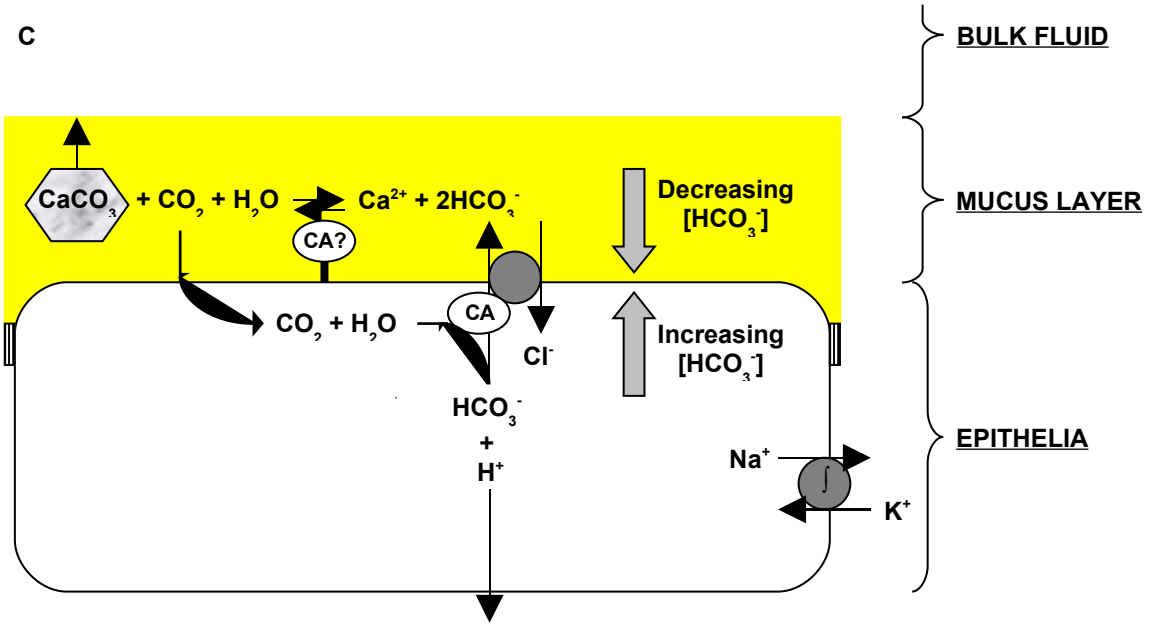



Figure 7.8: Panels A and B show transmission electron micrographs of the apical portion of the intestinal epithelium from the seawater-adapted European eel which has been incubated with lanthanum as an electron-dense tracer for $Ca^{2+}$ (Reproduced from Humbert *et al.*, 1989). (A) Mucus globules exiting from the goblet cell (gc), demonstrates how $Ca^{2+}$-becomes concentrated in the mucus as soon as it is extruded from the epithelium (x 8,000. Bar = 1 µm). (B) Needle-like $Ca^{2+}$-rich crystals are clearly visible within the overlying mucus layer along with very fine precipitates deposited on the microvilli (mv) (x 8,000. Bar = 2 µm). (C) A simple schematic diagram illustrating the potential role for $CaCO_3$ crystallisation in $HCO_3^-$ secretion, in conjunction with a $Cl^-/HCO_3^-$ exchanger which is linked to carbonic anhydrase (CA) as part of a transport metabolon, helping create a favourable gradient for $HCO_3^-$ secretion within the localised micro-environment at the apical membrane of the epithelial cell.

Interestingly, the failure of receptor agonists $Gd^{3+}$ and neomycin to stimulate $HCO_3^-$ secretion during perfusion of the intestine (Figure 7.6) would actually favour the notion that the rate of precipitation will determine what additional $HCO_3^-$ secretion takes place and not the CaR, yet by no means can this be considered definitive. The application of $Gd^{3+}$ as an agonist was perhaps not the most appropriate choice for use within the intestinal lumen which was rich in $HCO_3^-$ and $CO_3^{2-}$, since this trivalent lanthanide will easily form

complexes with these anions (Caldwell *et al*., 1998), and is likely to have become incorporated into the $CaCO_3$ precipitates as $Gd_2(CO_3)_3$. However, the rate of precipitation in the presence of $Gd^{3+}$ was actually 20 % lower than the control, and was reduced by almost one third with neomycin, although neither result was statistically significant (Figure 7.6). In addition, there was also a reduction in the proportion of fluid absorbed by one third (from 47.2 % to 33.3 % in the neomycin and control treatments, respectively) which was almost significant. There were no differences in the simultaneous rates of $Na^+$ and $Cl^-$ transport that could help explain this change in fluid absorption. Figure 7.10 reveals little difference in net $Na^+$ absorption between treatments ($F_{2, 13} = 0.39$, $P = 0.684$), although net $Cl^-$ absorption in the presence of neomycin was more than 20 % lower than the control but was not close to significance ($F_{2, 13} = 1.01$, $P = 0.390$).

The sample sizes of experiments involving these agonists were small ($n = 4$) compared to the control, which may preclude the detection of statistically significant differences, yet there are trends in the data suggesting potential differences. However, it was not clear whether this would have been a direct result of neomycin acting upon the CaR, particularly since its application *in vitro* did not have a convincing effect on ion and fluid transport (Figure 7.5). For example, neomycin is a widely used aminoglycoside antibiotic, which is included in oral solutions as part of the pre-operative prophylactic treatment of the gut prior to surgery (Nichols *et al*., 2005). Interestingly, Walsh *et al*.

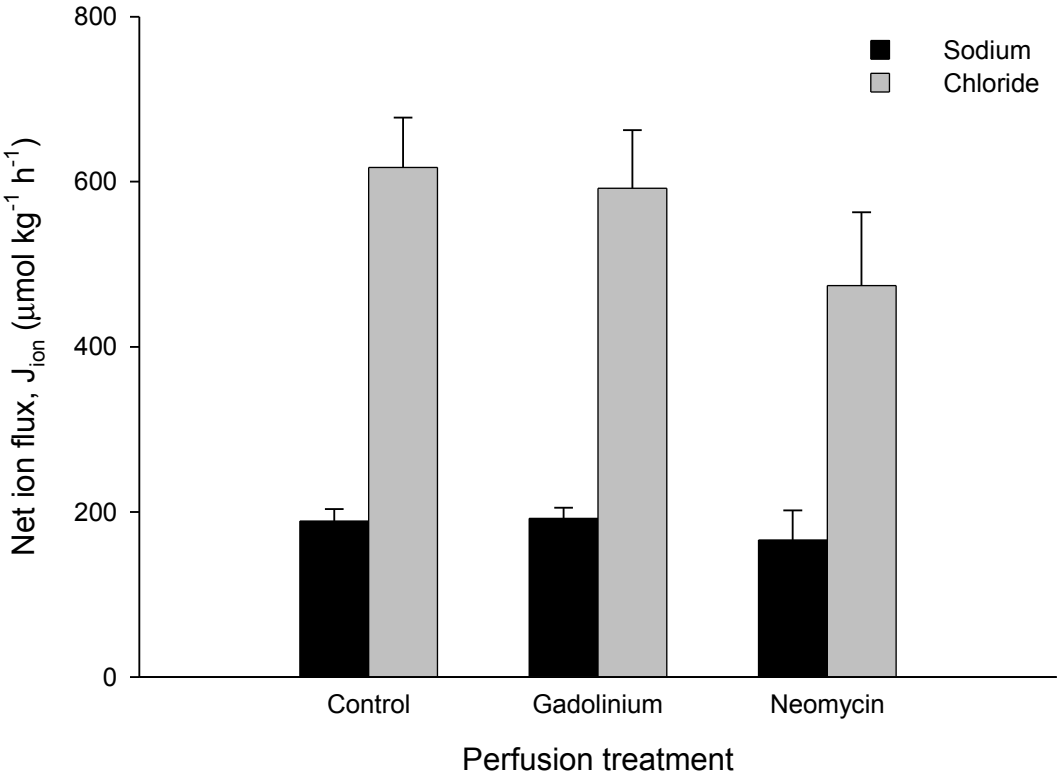



Figure 7.9: The mean (±SE) net sodium (open bars) and chloride (shaded bars) fluxes (µmol $kg^{-1}$ $h^{-1}$) by the intestine of the flounder perfused with salines containing the CaR agonists $Gd^{3+}$ and neomycin compared with the control over a total of 3 days (n = 8, 4 and 4 for the control, $Gd^{3+}$ and neomycin treatments, respectively).

(1991) concluded that carbonate precipitation by the Gulf toadfish does not involve intestinal microbes and combined application of the antibiotics, sisomycin and ampicillin (at a dose of 50 ppm each), reduced bacterial counts per intestine more than 1,000-fold (from $7.7 \times 10^7$ to $2.2 \times 10^4$), with no effect on the rate of $CaCO_3$ production. The amount of neomycin used in the current perfusion experiments was 10 times greater than the total antibiotic dose administered by Walsh *et al*. (1991). Since there is still much to learn about the actual process of $CaCO_3$ formation by the teleost intestine, if these trends for reduced precipitation and fluid absorption in the presence of neomycin (Figures 7.6 and 7.7), were proven beyond reasonable doubt, then it would open up the possibility that there may

indeed be a role for the microflora of the gut in $CaCO_3$ production, particularly since bacteria are capable of forming carbonate minerals (Boquet *et al*., 1973; Novitsky, 1981).

### 5.3 A role for the CaR *in vitro*

In the Ussing chamber where the process of $CaCO_3$ crystallisation is unlikely, given such low concentrations of $HCO_3^-$ and $CO_3^{2-}$, and with a sustained gradient for $HCO_3^-$ secretion, the tissue is able to respond to elevated mucosal $Ca^{2+}$ by increasing the rate of $HCO_3^-$ secretion to (presumably) encourage $CaCO_3$ formation and removal of the excess $Ca^{2+}$, with this process most likely being mediated *via* the CaR (Wilson *et al*., 2002). However, with no observed increases in $HCO_3^-$ secretion in response to $Ca^{2+}$ in the gut sac preparation (Figures 7.1 to 7.3), it was proposed that in order for the tissue to effectively deal with this $Ca^{2+}$ challenge the epithelium reduces fluid absorption, in some cases leading to secretion. This would not only help to prevent $Ca^{2+}$ concentrating at the apical membrane, but the inhibition of NaCl absorption would lead to hyper-polarisation of the membrane closing any voltage-gated, L-type $Ca^{2+}$ channels and thus further reducing the opportunity for entry of $Ca^{2+}$ into the tissue, helping to preserve $Ca^{2+}$ homeostasis (Chapter 4, Section 5.3.4). Since any increase in $HCO_3^-$ secretion in gut sacs has been undermined by the constraints of the technique, the demonstration of net fluid and NaCl secretion, under high $Ca^{2+}$ conditions (Figures 7.2 and 7.3), help support this hypothesis. The effect of elevated $Ca^{2+}$ concentration in Figures 7.2 and 7.3 is consistent with how the CaR operates in other transporting epithelia (Sands *et al*., 1997; Bruce *et al*., 1999; Fellner and Parker, 2002; Nearing *et al*., 2002; Geibel *et al*., 2006; Gerbino *et al*., 2007), and it was subsequently proposed that these responses may also be under control of the receptor. However, attempts to demonstrate the control of intestinal ion and fluid transport using agonists of the CaR, $Gd^{3+}$ and neomycin were not very convincing (Figures 7.4 and 7.5).

For $Gd^{3+}$, while $HCO_3^-$ was significantly reduced in the anterior and mid sections, accompanied by dramatically lower fluid transport rates (but no secretion), these were not recoverable after rinsing (with the possible exception of the posterior sections), hence they were contrary to the reversible nature of the response expected if $Gd^{3+}$ were operating *via* the CaR (Nearing *et al*., 2002). Furthermore, there was no evidence to suggest that NaCl co-transport was being adversely affected, in fact experiments with the reduced ionic

strength saline were characterised by consistent net $Na^+$ secretion, while rates of net $Cl^-$ transport were conspicuously low (Figures 7.2, 7.4 and 7.5).

Aside from the avid binding of $Gd^{3+}$ to anions such as $HCO_3^-$ and $CO_3^{2-}$, this trivalent lanthanide is also a potent blocker of $Ca^{2+}$ channels in a range of cells (Boland *et al*., 1991). For example, $Ca^{2+}$ channels of cardiac myocytes from the guinea pig were completely blocked by $Gd^{3+}$ displaying an $EC_{50}$ of just 1.4 µM (Lacampagne *et al*., 1994). In addition, 5 µM completely inhibited L-type $Ca^{2+}$ currents in rat pituitary cells (Biagi and Enyeart, 1990), and partial inhibition of L-type channels was observed with 10 and 50 µM $Gd^{3+}$ in ventricular myocytes from the rat (Sadoshima *et al*., 1992). Overall, since NaCl co-transport (and the putative $Ca^{2+}$ channels) were considered to be the intended targets in gut sacs following activation of the CaR with $Gd^{3+}$ (as well as neomycin), the experimental evidence presented in Figures 7.4 and 7.5 were not consistent with a role for the CaR.

### 5.4 Is $Cl^-/HCO_3^-$ exchange a driving force for fluid transport *in vitro*?

A rather puzzling aspect of the data presented in Figures 7.2 to 7.5, that deserves attention is how net fluid absorption was able to take place even though there was substantial net secretion of $Na^+$, and relatively little net $Cl^-$ transport. In these experiments fluid cannot be driven by a transepithelial differences in osmotic pressure, and must therefore be coupled to ion transport, yet fluid absorption from mucosa to serosa persisted in the apparent absence of net NaCl co-transport, the principal driving force behind fluid absorption *in vitro* and *in vivo*.

An unfortunate oversight of experiments using the reduced osmolality saline was that these conditions did impose an osmotic challenge on the tissue. This saline exerted an osmotic pressure of ~280 mOsm $kg^{-1}$, approximately 40 mOsm $kg^{-1}$ lower than what the intestine was likely to be experiencing *in vivo* (~320 mOsm $kg^{-1}$) prior to dissection. Hence, the cells would have been initially hypertonic to the bathing media, and as such this would have drawn water into the tissue. These gut sac preparations would therefore have benefited from a period of adjustment (discussed in Chapter 3, Section 5.1), allowing the tissue to adapt to these new conditions, before commencing the control incubation. Henceforth, these results are not referred to as part of the following discussion.

From very similar gut sac experiments with flounder, Grosell *et al*. (2005) also found that net $Na^+$ transport was unexpectedly characterised by secretion, yet observed a strong

correlation between net $Cl^-$ absorption and fluid transport suggesting that this provided (part of) the driving force, and presumably involved $Cl^-/HCO_3^-$ exchange. Similarly, $Cl^-$ absorption persisted alongside $HCO_3^-$ secretion in gut sacs from the intestine of the lemon sole (*Parophrys vetulus*) when presented with $Na^+$-free bathing solutions, and in addition to displaying sensitivity to mucosal DIDS, suggested $Cl^-/HCO_3^-$ exchange as a driving

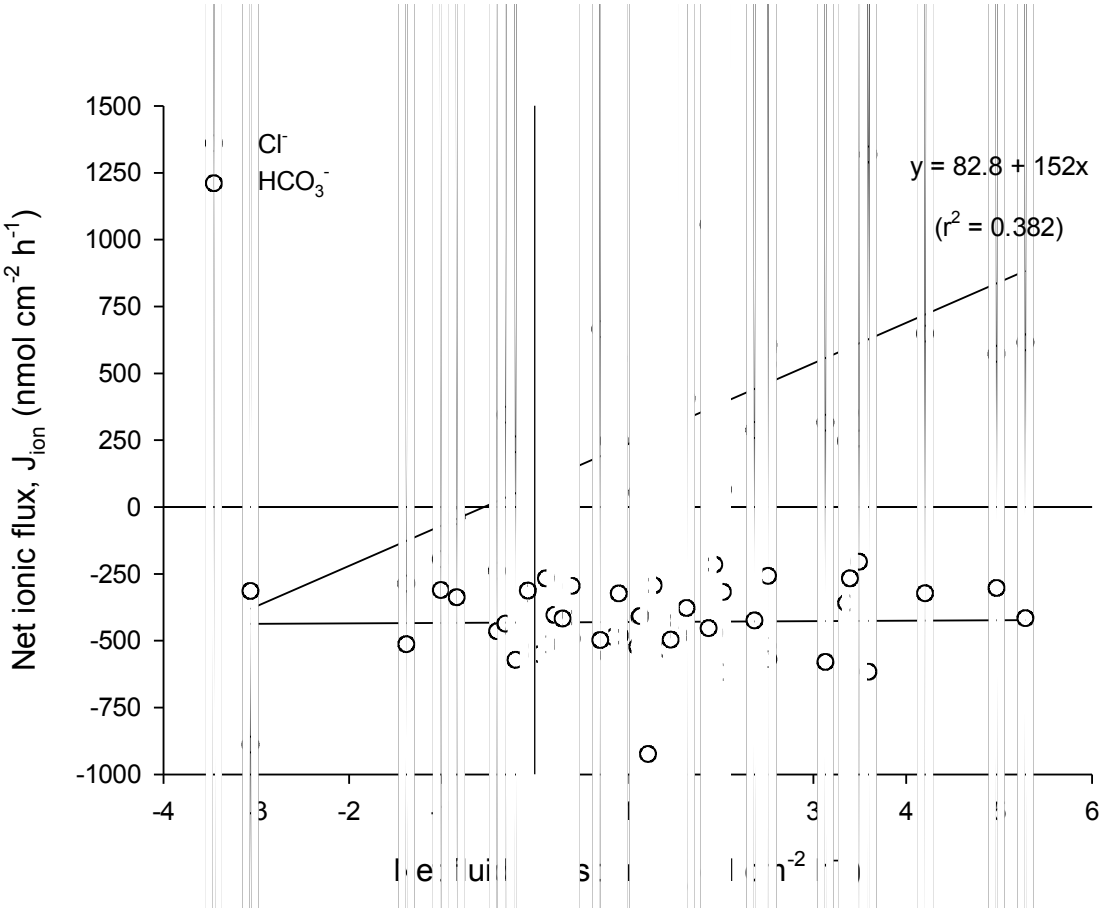



Figure 7.10: The net fluxes (nmol $cm^{-2}$ $h^{-1}$) of $Cl^-$ and $HCO_3^-$ in relation to net fluid transport (µl $cm^{-2}$ $h^{-1}$) using the overall mean values pooled from the anterior, mid and posterior gut sacs from all control incubations in the present study employing the reduced ionic strength mucosal saline (from Figures 2, 4 and 5) (n = 54).

force for fluid absorption (Grosell *et al.*, 2001). However, contrary to these conclusions, Figure 7.10 shows that the measured rate of $HCO_3^-$ secretion in the present study was very much independent of fluid transport (R = 0.021, P = 0.882), but there was a significant relationship with net $Cl^-$ flux ($F_{1, 52}$ = 32.16, P = <0.001). This would therefore make $Cl^-/HCO_3^-$ exchange an unlikely driving force for fluid absorption in the present study.

Having previously considered the thermodynamicsbehind $HCO_3^-$ secretion (Section 5.1), do the varying mucosal saline conditions presented to the tissue impose constraints on other ion fluxes? Examination of the electrochemicalprofiles for $Na^+$, $K^+$ and $Cl^-$ (Table 7.5) indicates that apical NaCl cotransport remains feasible even at mucosal $Na^+$ concentrations as low as 50 mM. However, under these latter conditions the overall direction of the transepithelial electrochemical driving force acting on $Na^+$ is reversed (from -0.28 to 1.13 kJ $mol^{-1}$), thus favouring a net entry of this ion into the mucosal saline. This is reflected by the accompanying measured fluxes of $Na^+$ which change from net absorption (504 ±176 nmol $cm^{-2}$ $h^{-1}$) with the 'regular' mucosal saline (containing 90 mM $Na^+$) to net secretion (-549 ±104 nmol $cm^{-2}$ $h^{-1}$) with the 'reduced ionic strength' saline.

The apparent net secretion of $Na^+$ into the mucosal saline observed in Figures 7.2to 7.5 is therefore likely to be passive, a consequence of the asymmetrical concentration of this ion in the bathing solutions. As $Na^+$ is pumped into the lateral intercellular space by $Na^+$-$K^+$-ATPase (providing the energy for apical NaCl cotransport), it will leak back into the mucosal saline, presumably across the tight junction (Table 5). Field *et al.* (1979) made a similar suggestion for the intestine of the winter flounder (*Pseudopleuronectes americanus*) to try and explain the preponderance of net $Cl^-$ flux over net $Na^+$ flux, characteristic of marine teleost epithelia. Further evidence that the distribution of $Na^+$ across the gut sac influences net $Na^+$ flux and subsequently fluid absorption is demonstrated in Figure 7.11. A comparison of measured $Na^+$ flux rates by gut sacs from the European flounder reveals a significant linear relationship with the concentration of $Na^+$ present in the mucosal saline ($F_{1,4} = 197.21$, $P = <0.001$). Interestingly, the predicted net $Na^+$ flux in relation to the concentration of mucosal $Na^+$ crosses the x-axis at

Table 7.5: A comparison of the electrochemical potentials, $\Delta\mu$ (kJ mol$^{-1}$) for $Na^+$, $K^+$ and $Cl^-$ as well as apical NaCl co-transport, for the 'regular' and 'reduced ionic strength' mucosal salines used with gut sacs from the flounder. Where $\Delta\mu$ is <0 transport will be thermodynamically feasible and the direction of ion movement along its electrochemical gradient is indicated in parentheses (where, m = mucosa, c = cytoplasm and s = serosa). Accompanying this analysis are the mean (±SE) net fluxes of each ion (nmol cm$^{-2}$ h$^{-1}$).

| **Ion** | **Concentration** (mM) | | | **Δμ** (kJ mol$^{-1}$) | | | **Apical cotransport Δμ** (kJ mol$^{-1}$) | | **Overall net flux*** (nmol cm$^{-2}$ h$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | **Mucosal** | **Cytoplasm** | **Serosal** | **Apical** | **Basolateral** | **Transepithelial ($\Delta\mu^{apical} + \Delta\mu^{basolateral}$)** | **$Na^+$-$K^+$-2$Cl^-$** | **$Na^+$-$Cl^-$** | |
| 'Regular' mucosal saline | | | | | | | | | |
| $Na^+$ | 91 | 15 | 155 | -13.91 (m→c) | 13.63 (c→m) | -0.28 (m→s) | -4.62 | -7.83 | 504 ±176 |
| $K^+$ | 5 | 87 | 3.5 | -2.89 (m→c) | -0.50 (c→s) | -3.39 (m→s) | | | -134 ±16 |
| $Cl^-$ | 135 | 30 | 153 | 6.09 (c→m) | -4.25 (c→s) | 1.84 (s→m) | | | 789 ±205 |
| 'Reduced ionic strength' mucosal saline | | | | | | | | | |
| $Na^+$ | 50 | 15 | 155 | -12.50 (m→c) | 13.63 (c→m) | 1.13 (s→m) | -2.41 | -6.01 | -549 ±104 |
| $K^+$ | 5 | 87 | 3.5 | -2.89 (m→c) | -0.50 (c→s) | -3.39 (m→s) | | | -50 ±17 |
| $Cl^-$ | 114 | 30 | 153 | 6.49 (c→m) | -4.25 (c→s) | 2.24 (s→m) | | | 55 ±138 |

*Average net fluxes from anterior, mid and posterior sections of the intestine (n = 6).

73.5 mM, almost the exact point at which $Na^+$ would be at electrochemical equilibrium across the tissue based on the Nernst equation (80.6 mM). Beyond this point fluid absorption continues but at a reduced rate and independent of associated net $Na^+$ transport (Figure 7.11).

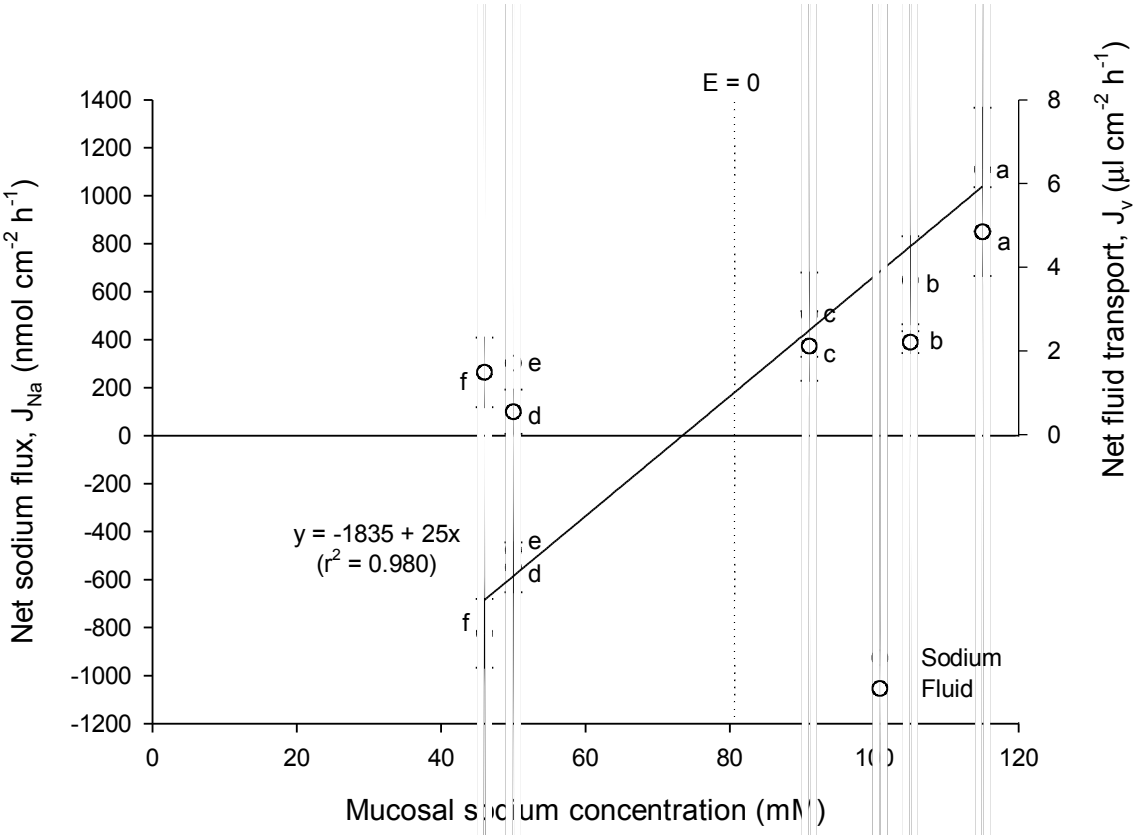



Figure 7.11: The relationships between net sodium flux, $J_{Na}$ (nmol cm$^{-2}$ h$^{-1}$), net fluid transport rate, $J_v$ (µl cm$^{-2}$ h$^{-1}$) and the concentration of $Na^+$ (mM) in the mucosal saline with gut sacs from the European flounder. The majority of the data was collated from previous chapters and presented as mean ±SE for the anterior, mid and posterior sections of the intestine combined: a) Chapter 2 (n = 10), b) Chapter 3 (n = 5), c) Chapter 4 (n = 6), d) Present study (reduced ionic strength, n = 6), e) Grosell *et al.* (2005) f) Present study (reduced osmolality, n = 6).

In summary, there was no evidence to suggest that in the absence of net $Na^+$ absorption, $Cl^-/HCO_3^-$ exchange significantly contributed to fluid absorption, contrary to previous *in vitro* observations (Grosell *et al.*, 2005). Examination of the electrochemical profiles for $Na^+$, $K^+$

and $Cl^-$ revealed that apical NaCl cotransport was still energetically feasible and would therefore be capable of driving fluid into the tissue, which would subsequently follow basolateral $Cl^-$ exit, while $Na^+$ passively recycled back into the mucosal saline thus accounting for the net negative $Na^+$ flux.

# Chapter Eight

## General discussion

The present study has not been able to demonstrate a direct role for $Ca^{2+}$-sensing receptors in the regulation of intestinal $HCO_3^-$ secretion in two model, euryhaline teleosts, the European flounder (*Platichthys flesus*) and killifish (*Fundulus heteroclitus*). Application of *in vitro* and *in vivo* techniques in Chapters 3 to 7, further exploring the fundamental characteristics of $HCO_3^-$ production, secretion and $CaCO_3$ precipitation by the intestine has already inspired detailed analysis and rather lengthy discussion on a variety of topics in and around this fascinating aspect of fish physiology. In this final section I have therefore decided to highlight two particularly interesting perspectives which merit further consideration and offer potential directions for future inquiry. The first of these follows on from the arguments put forth in Chapter 7 and presents a case for an alternate role for the CaR in relation to the regulation of $HCO_3^-$ secretion. Secondly, considering $HCO_3^-$ secretion and $CaCO_3$ precipitation as novel driving forces for fluid absorption, there is a short discussion of the potential implications for our current understanding of the nature of intestinal fluid transport by marine teleosts.

## 1. The $Ca^{2+}$-sensing receptor and regulation of intestinal $HCO_3^-$ secretion

Given the apparent limitations to measuring $HCO_3^-$ secretion when using the gut sac technique, (described in Chapter 7, Section 5.1), perhaps the most logical next step would have been to replicate the *in vitro* experiments performed by Wilson *et al*. (2002), utilising the Ussing chamber with the European flounder and apply agonists of the receptor, such as $Gd^{3+}$ and neomycin. On the face of it this would help to resolve the issue of whether the intestinal CaR is actually involved in $HCO_3^-$ secretion. However, if these experiments revealed that $HCO_3^-$ secretion were stimulated by CaR agonists, this could prove misleading, particularly if the data were interpreted as support for the hypothesis that the CaR directly regulates $HCO_3^-$ secretion by the teleost intestine. The inability to carry out such an experiment (as the Ussing chamber equipment was not available at Exeter, and flounder were not available in Miami), combined with the failure of $Ca^{2+}$ to stimulate $HCO_3^-$ secretion *in vitro*, using either the gut sac technique with the flounder (Chapters 4 and 7), or Ussing chamber with the killifish (Chapter 6), have forced a re-think of this original suggestion. Consequently, the proposal that $HCO_3^-$ secretion may not be modulated

by an intestinal CaR after all, but determined by the accompanying rates of intracellular $HCO_3^-$ production and luminal $CaCO_3$ precipitation (Chapter 7, Section 5.2.1) is an interesting one.

Widespread expression of the CaR along the gastrointestinal tract (Chapter 1, Section 4.3.1), and potentially fascinating links with digestion, $Ca^{2+}$ homeostasis and epithelial renewal (reviewed in Chapter 7, Section 2), offer plenty of scope for a direct, functional role for the receptor in the gut of teleost fish. Furthermore, there can be no question that the intestine has the capacity to directly and rapidly respond to elevated luminal $Ca^{2+}$ by increasing $HCO_3^-$ secretion, and this could be *via* a CaR (Wilson *et al*., 2002). However, is involvement of the receptor in regulating $HCO_3^-$ secretion a largely redundant function? The *in vitro* experiments performed by Wilson and co-workers took place under mucosal conditions imposed by the pH stat titration which are quite uncharacteristic for a teleost in seawater (pH 7.8, with $HCO_3^-$, $CO_3^{2-}$ and $CO_2$ all close to zero), thus providing a favourable gradient for $HCO_3^-$ secretion from serosa to mucosa. Perhaps a more appropriate experiment would have been to set the pH stat to a higher pH value (8.2-8.5), thus imposing more *in vivo*-like conditions across the tissue, before testing the effects of $Ca^{2+}$ on $HCO_3^-$ secretion.

Yet, if there were no effect of mucosal $Ca^{2+}$ *in vitro* when imposing a more realistic mucosal pH and $HCO_3^-$ gradient as suggested above, it could still be argued that involvement of an intestinal CaR in mediating $HCO_3^-$ secretion would be of considerable relevance for euryhaline species, such as the European flounder. For example, when moving from freshwater to seawater, stimulation of the drinking reflex and therefore entry of $Ca^{2+}$-rich fluid into the intestine will presumably demand a rapid response in terms of $HCO_3^-$ secretion. However, inconsistent with this argument is the lack of evidence for involvement of a CaR in the stimulation of $HCO_3^-$ secretion by the killifish intestine, another strongly euryhaline species (Chapter 6), thus suggesting there is indeed an alternate (or perhaps species-specific) mechanism regulating the secretion of $HCO_3^-$. Furthermore, there was an unexpected, dramatic 12-fold increase in $HCO_3^-$ concentration measured in the intestinal fluid of the toadfish (*Opsanus beta*) *in vivo* when acclimated to 5 ppt (McDonald and Grosell, 2006) compared with 2.5 ppt (Figure 8.1).

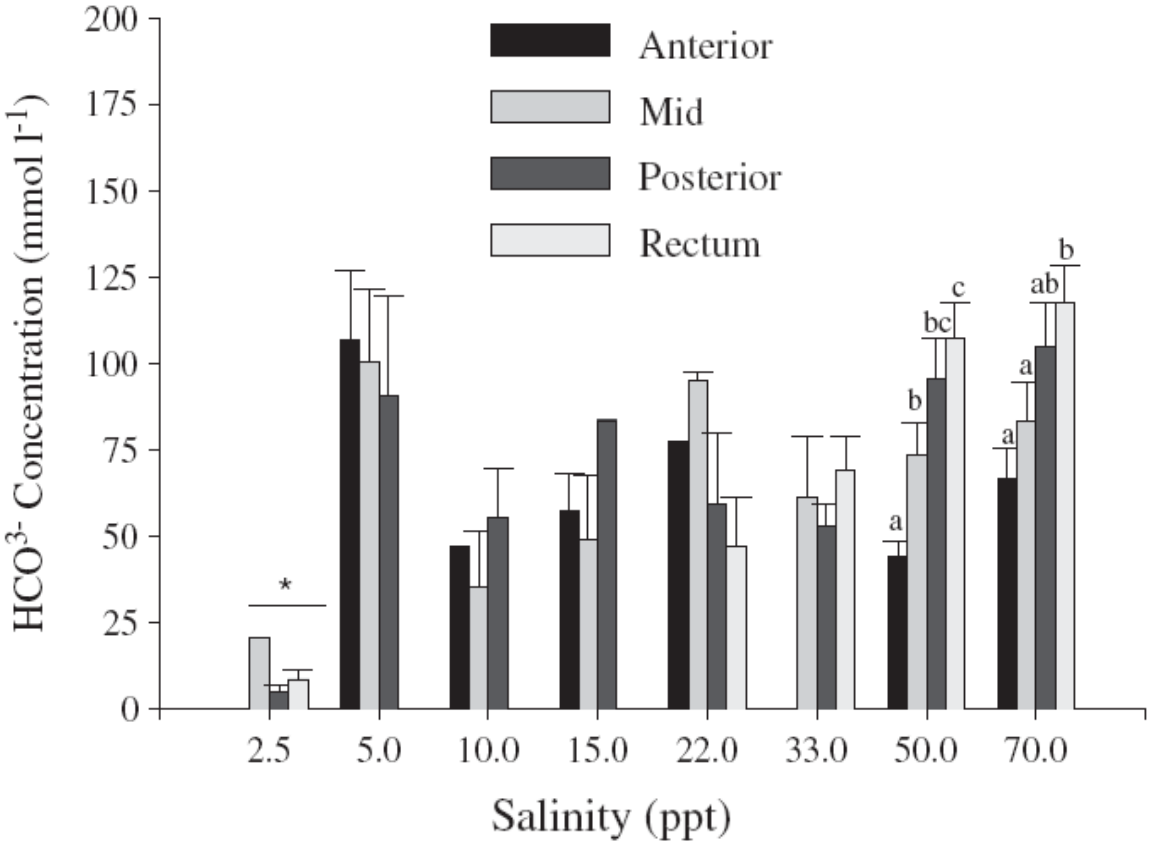



Figure 8.1: The concentration of bicarbonate (mM)in the anterior, mid, posterior and rectal fluids sampled from the intestine of the Gulf toadfish (*Opsanus beta*) acclimated to a range of salinities from 2.5 to 70 ppt. Values are means ±SE (n = 2-8). An asterisk denotes a statistically significant difference in the average intestinal fluid value from 33 ppt, and different letters indicate a significant difference between intestinal segments within a particular salinity. In all cases $P < 0.05$ (Reproduced from McDonald and Grosell, 2006).

While the data in Figure 8.1 does not directly provide any information on rates of secretion, it does imply that there is a significant increase in $HCO_3^-$ secretion at low salinities. If a role for the intestinal CaR is unlikely, even in euryhaline species, this naturally raises the question of what regulatory mechanismis behind the stimulation of $HCO_3^-$ secretion. Interestingly enough, the answer could still be the CaR, but involving activation of the receptor in the olfactory epithelium as opposed to the intestine. At 5 ppt the osmolality of the surrounding water will be around 160 mOsm $kg^{-1}$, compared to a measured plasma osmolality of 280 mOsm $kg^{-1}$ (McDonald and Grosell, 2006). The fish were therefore

distinctly hyperosmotic to the surrounding water, and faced a passive gain of water and loss of salts, consequently drinking rate would have been very low, or possibly absent altogether. Hence, it is difficult to associate a surge in $HCO_3^-$ secretion with activation of the intestinal CaR *via* imbibed $Ca^{2+}$, particularly since the intermediate salinities (10 to 33 ppt) did not produce equivalent or even higher intestinal $HCO_3^-$ concentrations in a $Ca^{2+}$-dependent manner (Figure 8.1). Since drinking rate is positively correlated with external salinity (Chapter 1, Section 2.1), it should be noted that the low volume of fluid expected within the intestine at 5 ppt, compared to full strength seawater (33 ppt) and at hypersalinities (50 and 70 ppt) where the drinking rate will be many times greater, could have also contributed to the very high concentrations of $HCO_3^-$ observed at such a low salinity. However, what is most intriguing about this data set is that the difference in $Ca^{2+}$ concentration between 2.5 and 5 ppt would only be ~0.7 mM (0.76 and 1.52 mM, respectively). This is almost identical to the $IC_{50}$ for the firing rate of the olfactory nerve of the euryhaline sea bream (*Sparus aurata*) in response to changes in environmental $Ca^{2+}$ concentration, which was determined as 1.67 ±0.26 mM (Hubbard *et al*., 2000). Furthermore, it has subsequently been proposed that the sensitivity of the olfactory system observed in teleosts is conferred by an extracellular CaR (Hubbard *et al*., 2000; 2002). These observations are indirectly supported by localisation of the receptor to the olfactory epithelia from a number of euryhaline teleosts (Naito *et al*., 1998; Flanagan *et al*., 2002; Nearing *et al*., 2002; Loretz *et al*., 2004; Dukes *et al*., 2006; see Chapter 7, Section 2.2 for more detailed discussion), consistent with purported roles in salinity adaptation (Nearing *et al*., 2002; Loretz, 2008). Distribution of the CaR in the nervous and endocrine systems of teleosts has also led to speculation on involvement of the receptor in a number of homeostatic functions, including osmoregulation (Chapter 1, Section 4.3.1). However, the physiological responses to olfactory signals indicating changes in environmental $Ca^{2+}$ have yet to be explored. It was originally suggested that this could be a component of the homing migrations made by salmon (Bodznick, 1978). Yet more pertinently, this would signal activation of the appropriate physiological processes to cope with changes in salinity (Hubbard *et al*., 2000), and more specifically this could extend to the regulation of intestinal $HCO_3^-$ secretion.

## 2. Fluid absorption by the marine teleost intestine

### 2.1 Absorbing a hyperosmotic fluid

The goal of the osmoregulatory strategy of marine teleosts is to not only conserve water, but by doing so maintain the appropriate balance of water and salts within the body. In addition to NaCl absorption, the production and secretion of $HCO_3^-$ has a fundamental role in helping the body cope with imbibed seawater and more effectively absorb fluid from the intestine. It is therefore a source of curiosity as to why this absorbed fluid appears to be hyperosmotic in nature, based on calculations from the net fluxes of ions from the intestinal lumen along with associated fluid movements (Grosell, 2006; Grosell and Taylor, 2007). Similar findings were also confirmed for the European flounder from calculations utilising the *in vivo* perfusion data in Chapter 5 (Section 5.5.1). Absorption of a hyperosmotic fluid by the intestinal epithelia is considered an unavoidable consequence of $HCO_3^-$ secretion and basolateral $H^+$ secretion, in conjunction with reduced levels of NaCl, corresponding with high concentrations of $MgSO_4$, in the luminal fluid (Grosell and Taylor, 2007).

### 2.2 The influence of basolateral $H^+$ secretion

The polarity of apical $HCO_3^-$ secretion and basolateral $H^+$ extrusion has since been demonstrated *in vitro* (Grosell and Genz, 2006; Grosell and Taylor, 2007; Chapter 4, Section 4.3), as well as (indirectly) *in vivo* (Genz *et al.*, 2008; Chapter 5, Section 5.4). Perfusion of the intestine with saline containing 90 mM $Ca^{2+}$ produced up to an 8-fold increase in the rate of $HCO_3^-$ secretion matched almost exactly by an associated elevation in $H^+$ production, theoretically contributing to the osmolarity of the absorbed fluid. To resolve the observed paradox between the calculated hyperosmolarity of absorbed fluid and the measured reduction in blood plasma osmolality in these experiments, it was suggested that $H^+$ arising from intracellular $CO_2$ hydration could be buffered by extracellular $HCO_3^-$ to yield $CO_2$ concomitant with a reduction in osmotic pressure of the absorbed fluid (Chapter 5, Section 5.5). Consistent with these observations, if the osmolarity of absorbed fluid from *in vitro* preparations excluded the associated 'missing cation' (presumed to be $H^+$ and buffered by the extracellular fluids) this produces a substantial reduction in osmolarity, equivalent to an average difference of 69 mOsm $l^{-1}$ (Table 8.1). This further highlights the benefit of this proposed buffering process to the osmotic pressure of the fluid being

absorbed by the intestine. However, in some cases the absorbate remained hyperosmotic (426-611 mOsm $l^{-1}$), even after excluding the $H^+$ concentration, yet these calculations are confined to preparations where net fluid absorption has been measured gravimetrically, which can under-estimate the value of $J_v$ (Wilson and Grosell, in preparation; Chapter 2, Section 2.2), and consequently lead to the calculation of higher concentrations of $Na^+$ and $Cl^-$ in the absorbed fluid (Table 8.1). Interestingly, when compared with data cited from recent work using gut sac preparations from the toadfish (Grosell and Taylor, 2007; Wilson and Grosell, in preparation), where $J_v$ was calculated based on the marker $^{14}$C-PEG 4000, a distinctly hypoosmotic absorbate was produced (Table 8.1). Furthermore, it should also be remembered that the values for $J_v$ presented here for *in vitro* experiments will not have benefited from the additional fluid that would be absorbed as a result of $CaCO_3$ precipitation which occurs *in vivo*, contributing to a further reduction in absorbate osmolarity.

**2.3 Absorption of an iso-osmotic fluid**

Having considered this idea further, and based on data collected *in vivo* (Chapter 5) and *in vitro* (Table 8.1), it is submitted that the fluid absorbed by the marine teleost intestine under *in vivo*-like conditions is actually more likely to be isoosmotic, in common with fluid-transporting epithelia from other vertebrates, or perhaps even hypoosmotic. The challenge of coping with the intake of substantial amounts of divalent ions with seawater makes absorption of an isoosmotic fluid by the intestine dependent on the involvement of a number of unique processes and conditions. These include the production of $CaCO_3$

Table 8.1: The reduction in osmotic pressure of fluid absorbed by the teleost intestine, following the buffering of absorbed $H^+$ by the extracellular fluid. The concentrations (mmol $l^{-1}$) of $Na^+$ and $Cl^-$ in the absorbate were calculated from the net fluxes of $Na^+$, $Cl^-$ (µmol $cm^{-2}$ $h^{-1}$) and fluid (µl $cm^{-2}$ $h^{-1}$) by *in vitro* preparations of the intestine from a number of euryhaline teleosts under asymmetrical, *in vivo*-like bathing conditions in the absence of a transepithelial osmotic gradient. The shortfall of cations required for charge balance in the absorbed fluid were assumed to be $H^+$, and labelled 'missing $H^+$'. The predicted osmotic pressure of the absorbed fluid was assumed to have an osmotic coefficient of 0.94 (Robinson and Stokes, 1965). Net fluid absorption was measured gravimetrically, unless stated.

| | **Net flux** (µl or µmol $cm^{-2}$ $h^{-1}$) | | | **Concentration in absorbed fluid** (mmol $l^{-1}$) | | | **Osmotic pressure of absorbed fluid** (mOsm $l^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $J_v$ | $J_{Na}$ | $J_{Cl}$ | $Na^+$ | $Cl^-$ | 'Missing' $H^+$ | With $H^+$ | Without $H^+$ | Δ Osm. |
| European flounder[1] | 4.85 | 1.11 | 1.28 | 229 | 264 | 35 | 475 | 444 | 32 |
| [2] | 2.21 | 0.65 | 0.85 | 294 | 385 | 90 | 692 | 611 | 81 |
| [3] | 2.12 | 0.50 | 0.79 | 236 | 373 | 137 | 671 | 548 | 123 |
| Rainbow trout[4] | 4.72 | 0.47 | 1.08 | 100 | 229 | 129 | 412 | 296 | 116 |
| Gulf toadfish[5†] | 6.52 | 0.77 | 1.12 | 118 | 172 | 54 | 309 | 260 | 48 |
| [6] | 7.10 | 1.44 | 1.92 | 203 | 270 | 68 | 487 | 426 | 61 |
| [6†] | 4.81 | 0.54 | 0.70 | 112 | 146 | 33 | 262 | 232 | 30 |

[†]Net fluid transport ($J_v$) measured using the marker $^{14}$C-PEG 4000

[1]Chapter 2 (Figure 2.2); [2]Chapter 3 (Figure 3.2); [3]Chapter 4 (Figure 4.2); [4]Personal observations; [5]Grosell and Taylor (2007); [6]Wilson and Grosell (in preparation)

precipitates (Wilson *et al*., 2002), and retention of high concentrations of $MgSO_4$, the latter being characterised by a low osmotic coefficient (Taylor and Grosell, 2006a; Grosell and Taylor, 2007), together these will help reduce the osmotic pressure within the intestinal lumen and consequently benefit the absorption of additional fluid. Similarly, it has been speculated that low concentrations of $Ca^{2+}$ and/or elevated osmotic pressure within the lumen will activate apical $H^+$ secretion, with the effect of titrating mucosal $HCO_3^-$, which may also contribute to a reduction in the osmotic pressure of fluid within the gut (Grosell *et al*., 2007*; see also Chapter 4, Section 5.2.2 and Chapter 6, Section 5.4). Finally, and perhaps most importantly, the buffering of $H^+$ (secreted across the basolateral membrane) by extracellular $HCO_3^-$ producing $CO_2$ (which exerts a negligible osmotic pressure) will help further reduce the osmolarity of the hyperosmotic fluid being absorbed from the intestine (Chapter 5, Section 5.5.2). It would be interesting to further explore this idea and to what extent these various components interact (and potentially feedback on one another), to maintain the appropriate balance of fluid entering the body.

### 2.4 Further implications of intestinal $H^+$ production

From a broader perspective, the process of basolateral $H^+$ secretion by the intestine has recognised consequences for whole animal acid-base homeostasis where significant base excretion by the intestine will require a corresponding $H^+$ efflux, most likely *via* the gills (Wilson *et al*., 1996; Wilson and Grosell, 2003; Genz *et al*., 2008; Chapter 5, Section 5.4.1). The removal of $H^+$ by the intestine into the body in the form of $CO_2$ will subsequently need to be transported in the blood to the gills for excretion, and the hypothesis that elevated intestinal $HCO_3^-$ production and $CaCO_3$ precipitation will incur higher rates of respiratory $CO_2$ excretion are currently being addressed. Indeed, this may explain why branchial carbonic anhydrase (CA) expression and activity (which is not directly related to NaCl secretion by the gills) has been observed to increase following acclimation to seawater in a number of euryhaline teleosts (Kultz *et al*., 1992; Najib and

*It should be noted that this latter prediction by Grosell and co-workers (2007) for apical $H^+$ secretion would imply that the intestinal epithelia can indeed modulate $HCO_3^-$ secretion in response to luminal $Ca^{2+}$ (albeit indirectly, by shifting the direction of epithelial $H^+$ secretion). This could therefore prove a possible caveat to the suggestion that the intestinal CaR is essentially redundant in terms of regulating $HCO_3^-$ secretion.

Martine, 1996; Blanchard and Grosell, 2006; Boutet *et al*., 2006; Scott *et al*., 2008), thus helping to facilitate the excretion of this additional burden of $CO_2$. However, these observations do not currently extend to the European flounder where the levels of CA activity in the gills (Mashiter and Morgan, 1975), as well as the erythrocytes (Carter *et al*., 1976; Sender *et al*., 1999) were found to be independent of salinity. Similarly, CA expression by the gill epithelium remained unchanged following intestinal perfusion with 90 mM $Ca^{2+}$ (Cooper, C. A., personal communication).

## 3. Concluding remarks

After taking into consideration the discussion and analysis from the collected series of investigations presented here, there clearly remains much to learn about how the process of intestinal $HCO_3^-$ production and secretion is regulated, and this study has highlighted some fascinating avenues for future inquiry. The involvement of $CaCO_3$ precipitation, and differential rates of apical and basolateral $H^+$ secretion, along with the proposed role of an olfactory CaR, opens up the possibility that $HCO_3^-$ production and secretion is likely to be modulated through a combination of localised and environmental conditions. Although not fully understood, these potential regulatory mechanisms of $HCO_3^-$ secretion clearly function as a vital component to effective fluid absorption by the intestine, and ultimately overall body fluid balance in marine teleost fish.

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