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Review

Intestinal anion exchange in marine fish osmoregulation

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Summary

Despite early reports, dating back three quarters of a century, of high total CO_2 concentrations in the intestinal fluids of marine teleost fishes, only the past decade has provided some insight into the functional significance of this phenomenon. It is now being recognized that intestinal anion exchange is responsible for high luminal HCO_3^- and CO_3^{2-} concentrations while at the same time contributing substantially to intestinal Cl^- and thereby water absorption, which is vital for marine fish osmoregulation. In species examined to date, the majority of HCO_3^- secreted by the apical anion exchange process is derived from hydration of metabolic CO_2 with the resulting H^+ being extruded *via* a Na^+:H^+ exchange mechanism in the basolateral membrane. The basolateral H^+ extrusion is critical for the apical anion exchange and relies on the Na^+ gradient established by the Na^+-K^+-ATPase. This enzyme thereby ultimately fuels the secondary active transport of HCO_3^- and Cl^- by the apical anion exchanger. High cellular HCO_3^- concentrations (>10 mmol l^{-1}) are required for the anion exchange process and could be the result of both a high metabolic activity of the intestinal epithelium and a close association of the anion exchange protein and the enzyme carbonic anhydrase. The anion exchange activity *in vivo* is likely most pronounced in the anterior segment and results in net intestinal acid absorption. In contrast to other water absorbing vertebrate epithelia, the marine teleost intestine absorbs what appears to be a hypertonic fluid to displace diffusive fluid loss to the marine environment.

Key words: hypertonic water absorption, Cl^-/HCO_3^- exchange, secondary active Cl^- and HCO_3^- transport, acidic absorbate.

Osmoregulation in marine fish

Three distinct strategies for maintaining salt and water balance have evolved in fishes inhabiting the marine environments. (1) Osmoconformity/ionoconformity is found in the strictly marine agnathan hagfishes, which do not appear to regulate osmotic pressure and concentrations of main osmolytes to a great extent in seawater (Morris, 1958). (2) Osmoconformity and ionoregulation are seen in marine elasmobranchs (Hazon et al., 2003) and in the lobe-finned coelacanth (Griffith et al., 1974), which maintains plasma osmolality at or slightly above that of the surrounding seawater but NaCl concentrations at approximately ⅓ of ambient levels. (3) The most common strategy is osmoregulation, found in all teleosts (Marshall and Grosell, 2005) and marine lampreys (Morris, 1958), achieved by the regulation of the main extracellular electrolyte (Na^+ and Cl^-) levels at approximately ⅓ of full strength seawater.

Under steady state conditions at constant salinities, hagfish, elasmobranchs and coelacanths presumably do not need to drink to maintain water balance. It was shown, however, that the unavoidable renal and extra-renal fluid loss to the hypertonic marine environment in hypo-osmoregulating fish was compensated for by ingestion of seawater with subsequent water absorption across the gastro-intestinal tract (Smith, 1930). More recently, it was demonstrated that even the osmoconforming elasmobranchs display transient drinking when exposed to elevated ambient salinity, and evidence for components of the rennin–angiotensin system (RAS), even in the elasmobranchs (Anderson et al., 2002; Hazon et al., 2003) and hagfish (Cobb et al., 2004) as well as in lamprey (Brown et al., 2005), continues to accumulate. The drinking reflex is at least in part controlled by RAS and it thus appears that the ability to regulate ingestion of seawater and thereby the magnitude of intestinal fluid absorption is an ancestral osmoregulatory trait among fishes.

The ingestion and processing of the imbibed seawater for osmoregulatory purposes have, at least in teleost fish, received much attention for three quarters of a century since the first classic studies by Smith published in 1930 (Smith, 1930). It is now well established that an initial desalinization of the ingested seawater occurs in the esophagus, which absorbs Na^+ and Cl^- through both passive and active transport pathways,

although the cellular mechanisms remain to be fully elucidated. The esophageal absorption of salt combined with a limited water permeability (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983) of this gastro-intestinal segment results in a reduction of osmotic pressure in the fluids entering the anterior portion of the intestine (Marshall and Grosell, 2005). This reduction in osmotic pressure allows for fluid absorption by the more distal segments of the gastro-intestinal tract. Fluid absorption appears to occur along the entire length of the intestine at rates of 2–6 μl cm^{-2} h^{-1} (Grosell et al., 1999; Grosell et al., 2001; Grosell et al., 2005; Grosell and Jensen, 1999; Wilson et al., 2002) despite slight osmotic uphill gradients (McDonald and Grosell, 2006) and is driven by Na^+ and Cl^- transport (Ando et al., 1986; Mackay and Lahlou, 1980; Skadhauge, 1974; Usher et al., 1991). The preferential absorption of Na^+, Cl^- and water leaves Mg^{2+} and SO_4^{2-} highly concentrated in the intestinal fluids such that $MgSO_4$ accounts for the majority of the osmotic pressure (Grosell et al., 2001; Wilson et al., 2002). The chemical composition of the intestinal fluids is unusual in containing these high concentrations of divalent ions and also very high levels of HCO_3^-, and by being alkaline with pH in some cases reaching values higher than 9 (Wilson et al., 1996; Wilson, 1999).

Intestinal water absorption in marine teleost fish (and likely other marine animals required to drink seawater), is critical for water balance and occurs against an osmotic gradient. Water absorption occurs as a consequence of Na^+ and Cl^- transport, despite high luminal concentrations of divalent ions and alkaline conditions. The present review summarizes information about intestinal salt absorption, which provides the driving force for water transport. Discussion of whether water is absorbed through transcellular or paracellular routes and which water specific carrier proteins may play a direct role in water movement is beyond the scope of this text. Special emphasis will be on intestinal anion exchange, which has recently been attributed a significant role in Cl^- and water absorption by the marine teleost intestine (Grosell et al., 2005; Wilson et al., 2002). In addition, the net composition of the fluid absorbed by the marine teleost intestine is discussed as it appears to deviate from the isotonicity typical of water absorption across other vertebrate leaky epithelia, including the gall bladder, the renal tubule and small intestine of terrestrial vertebrates (Larsen, 2000; Larsen et al., 2002; Nedergaard et al., 1999).

Intestinal transport processes

NaCl absorption

The driving force: Na^+/K^+-ATPase

Water absorption across the marine teleost intestine is tightly linked to the absorption of Na^+ and Cl^- (Mackay and Janicki, 1978; Skadhauge, 1974; Usher et al., 1991). The Na^+ absorption is ultimately fueled by the basolateral Na^+/K^+-ATPase (NKA), which extrudes $3Na^+$ in exchange for $2K^+$ and thereby establishes a strong cytosolic negative membrane potential and low intracellular Na^+ concentrations (Skou, 1990; Skou and Esmann, 1992) (Fig. 1). The NKA activity is generally high in marine fish intestine, even when compared to the gill (Grosell et al., 1999; Hogstrand et al., 1999), and is higher in euryhaline fish acclimated to seawater than in freshwater acclimated individuals (Colin et al., 1985; Fuentes et al., 1997; Jampol and Epstein, 1970; Kelly et al., 1999; Madsen et al., 1994), illustrating the osmoregulatory importance of this enzyme. Furthermore, NKA gene

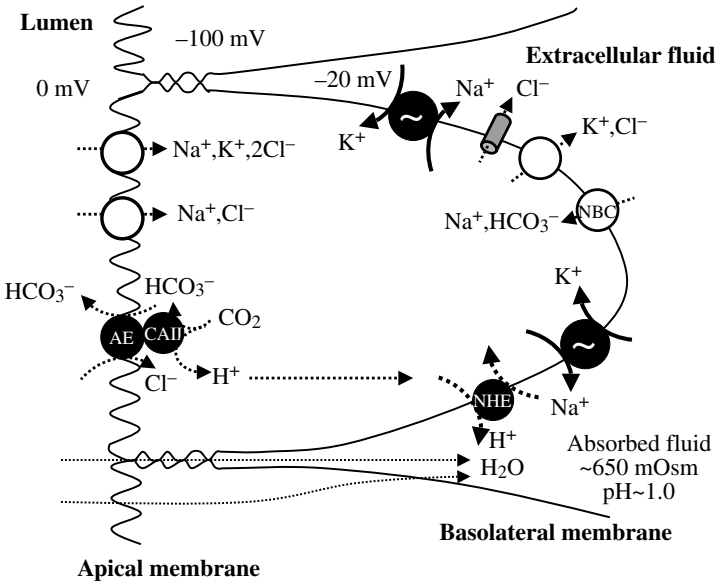

Fig. 1. Schematic cellular model of transport processes in the intestinal epithelium of marine teleost fish. Transcellular and/or paracellular fluid absorption is driven by active NaCl transport fueled primarily by the basolateral Na^+-K^+-ATPase (~), which provides the electrochemical Na^+ gradient allowing for Na^+, Cl^- and K^+ entry across the apical membrane. Two parallel systems, the Na^+,Cl^- and the Na^+,K^+,$2Cl^-$ cotransporters account for Na^+, K^+ and a portion of Cl^- absorption, with the remaining Cl^- uptake occurring *via* anion exchange (AE). The apical AE performs active transport of not only Cl^- but also HCO_3^-, resulting in high luminal HCO_3^- concentrations and highly alkaline intestinal fluids. Endogenous metabolic CO_2 provides cellular HCO_3^- *via* carbonic anhydrase for the apical anion exchange process, with the resulting H^+ being extruded across the basolateral membrane *via* an NHE-like transporter. The H^+ extrusion across the basolateral membrane is critical for apical HCO_3^- secretion and ultimately relies on the activity of the basolateral Na^+-K^+-ATPase. A physical association of AE and carbonic anhydrase II (CAII) might explain how local HCO_3^- concentrations on the luminal side of the apical membrane can reach levels satisfying the thermodynamical conditions necessary for anion exchange. Exchange of a metabolic waste product (CO_2), which exerts limited osmotic pressure, in exchange for an electrolyte provides an osmotic driving force for cellular water uptake. Basolateral import of HCO_3^- from extracellular fluids appears to also contribute to luminal HCO_3^- secretion and may occur *via* Na^+:HCO_3^- cotransport (NBC). Based on previous studies summarized in Table 2, fluid absorbed by the intestinal epithelium is hyper-osmotic and highly acidic (values represent means of all studies listed in Table 2). See text for further details.

expression is elevated in the intestine of euryhaline teleost fish following transfer from freshwater to seawater, demonstrating the significance of this enzyme for successful marine osmoregulation (Cutler et al., 2000; Jensen et al., 1998; Seidelin et al., 2000).

Apical co-transporters

The electrochemical Na^+ gradient established by the basolateral NKA provides the energy necessary for the thermodynamic uphill transport of K^+ and Cl^- from the intestinal lumen across the apical membrane *via* two parallel cotransport systems: Na^+:Cl^- (NC) and Na^+:K^+:2Cl^- (NKCC) co-transporters (Field et al., 1980; Frizzell et al., 1979; Halm et al., 1985; Musch et al., 1982) (Fig. 1). As for NKA, the NKCC gene exhibits increased expression in euryhaline fish intestine when transferred from freshwater to seawater (Cutler and Cramb, 2002), illustrating the importance of NKCC for seawater osmoregulation. A large number of studies have demonstrated that net Cl^- absorption rates exceed corresponding net Na^+ absorption rates (Table 1), which may in part be attributed to the stoichiometry of NKCC. However, considering the relatively low K^+ concentration in seawater (10 mmol l^{-1} compared to ~430 mmol l^{-1} Na^+), NKCC cannot account for the often large excess of net Cl^- absorption (see 'Missing cationic charge' section, below). Recently, much of the excess net Cl^- absorption has been linked to anion exchange processes in the apical membrane of the marine teleost intestinal epithelium (Grosell et al., 2005).

Anion exchange

Historical perspective and functional significance

High luminal total CO_2 concentrations and alkaline conditions (evident from the Phenol Red reaction) in intestinal fluids from three different marine teleost species were first reported 75 years ago and were found in both starved and fed fish (Smith, 1930), excluding a strictly digestive role of this phenomenon. Almost four decades later, the original observations by Smith were confirmed for rainbow trout acclimated to different salinities (Shehadeh and Gordon, 1969). Although total CO_2 was not measured directly in these studies on rainbow trout, a large anion:cation gap was observed by comparing concentrations of the major cations (Ca^{2+}, K^+, Mg^{2+} and Na^+) to concentrations of major anions (Cl^- and SO_4^{2-}) and was attributed to high concentrations of HCO_3^- and CO_3^{2-} (Shehadeh and Gordon, 1969). The study on rainbow trout reported direct measurements of pH in the intestinal or rectal fluids ranging between 8.1 and 9.0, depending on salinity

Table 1. *Net mean uptake rates of Na^+ and Cl^- across the intestinal epithelium in eight marine teleost fish*

Species	Net Cl^- flux	Net Na^+ flux	ΔNa^+–Cl^-	Net HCO_3^- flux	% of Cl^- flux
Parophrys vetulus[1,§]	0.4	0.2	–0.2	–0.2	50
Citharichthys dordidus[2,*]	3.5	2.3	–0.9	–0.9	26
Platichthys flesus[3,*]	1.0	0.8	–0.2	–0.3	30
Opsanus beta[4,†]	0.7	0.3	–0.4	–0.5	71
Platichthys flesus[5,†]	0.6	–0.4	–1.0	–0.3	50
Anguilla anguilla[6,‡]	3.0	0.9	–2.1	–	(70)
Pseudopleuronectes americanus[7,‡]	3.1	1.2	–1.9	–	(61)
Pseudopleuronectes americanus[8,‡]	5.5	2.0	–2.5	–	(45)
Platichthys flesus[9,‡]	6.5	2.2	–4.3	–	(66)
Platichthys flesus[10] (FW)[‡]	15.8	9.1	–6.7	–	(42)
Platichthys flesus[10] (SW)[‡]	26.6	18.5	–8.1	–	(30)
Platichthys flesus[11] (FW)[‡]	1.7	1.1	–0.6	–	(35)
Platichthys flesus[11] (SW)[‡]	2.5	1.7	–0.8	–	(32)
Platichthys lethostigma[12]	2.1	1.9	–0.2	–	(10)
Gillichthys mirabilis[13,‡]	2.5	1.3	–1.2	–	(48)

Flux values are in mequiv kg^{-1} h^{-1} or μequiv cm^{-2} h^{-1}.

FW, freshwater; SW, seawater.

ΔNa^+–Cl^- transport shows the difference between Na^+ and Cl^- net absorption. For four of the eight species represented, net HCO_3^- secretion (negative net flux) was also determined. For these four species, the magnitude of net HCO_3^- secretion is similar to the ΔNa^+–Cl^- transport, suggesting that Cl^- uptake, in excess of Na^+ uptake, is driven by HCO_3^-/Cl^- exchange. The column labeled '% of Cl^- flux' shows the fraction of Cl^- uptake that may be accounted for by anion exchange based on simultaneous measurements of HCO_3^- secretion. Values in parentheses are calculated assuming that ΔNa^+–Cl^- can be accounted fully for by anion exchange.

[†]Transport by isolated epithelia under asymmetrical conditions in absence of an osmotic gradient.

*Transport by isolated epithelia under asymmetrical conditions with osmotic pressure in serosal fluids>mucosal fluids.

[‡]Transport by isolated epithelia under symmetrical conditions.

[§]*In situ* perfusion.

1 (Grosell et al., 1999); 2 (Grosell et al., 2001); 3 (Grosell and Jensen, 1999); 4 (M.G., unpublished); 5 (Grosell et al., 2005); 6 (Marvao et al., 1994); 7 (Field et al., 1980); 8 (Musch et al., 1990); 9 (Mackay and Lahlou, 1980); 10 (Smith et al., 1975); 11 (Gibson et al., 1987); 12 (Hickman, 1968); 13 (Loretz, 1996).

and from which segment of the intestine the fluid samples were obtained. These pH values agree well with the first reports of pH increasing in rectal fluids from 7 to 9 in eels during seawater acclimation (Cordier and Maurice, 1956). It was recognized (Shehadeh and Gordon, 1969) that the likely source of HCO_3^- and CO_3^{2-} was endogenous and further, that Cl^-/HCO_3^- exchange might account for both secretion of HCO_3^- and absorption of Cl^-. Observations of a higher carbonate content in intestinal fluids and higher intestinal Cl^- absorption rates in trout acclimated to higher salinities (Shehadeh and Gordon, 1969) in these early studies had already suggested a role for Cl^-/HCO_3^- exchange in marine osmoregulation. In the first study of the mechanisms by which HCO_3^- is secreted into the intestine, a direct requirement for Cl^- as well as DIDS sensitivity was demonstrated in the goby and it was suggested that basolateral Cl^-/HCO_3^- exchange might assist in Cl^- transport across the basolateral membrane (Dixon and Loretz, 1986). These findings using the goby also implied a role for intestinal anion exchange in marine osmoregulation. Subsequently, evidence for an apical, DIDS sensitive Cl^-/HCO_3^- exchange process in the seawater acclimated eel intestine (Ando and Subramanyam, 1990) further supported a role for anion exchange in intestinal Cl^- absorption. Also supporting the role for intestinal anion exchange in osmoregulation were observations of rectal secretion of solid carbonate pellets (see 'Alkaline precipitation' section below) in gulf toadfish held in 100% seawater but not in 25% seawater (hypo-osmotic) (Walsh et al., 1991). In agreement with Shehadeh and Gordon (Shehadeh and Gordon, 1969), Walsh and co-workers concluded from their investigation of the gulf toadfish that the source of total CO_2 found in the intestinal lumen of toadfish was most likely endogenous, and further, that intestinal epithelial respiration was the likely source of CO_2. This latter suggestion was not empirically tested until much later (see 'Mechanisms of intestinal anion exchange' section below), but was supported by the high density of mitochondria observed in the intestinal epithelial cells (Walsh et al., 1991), indicating a high level of CO_2 production.

Lastly, pH measurements of rectal fluids obtained from coho salmon following seawater transfer revealed highly alkaline rectal fluids in smolts just 24 h post seawater transfer (Kerstetter and White, 1994). This was in contrast to post-smolt individuals where rectal fluids were circum-neutral 24 h post transfer and remained less alkaline than fluids obtained from smolts until 7 days post transfer, at which time rectal fluids from both smolts and post-smolts were highly alkaline.

Recent demonstrations of intestinal HCO_3^- secretion in elasmobranchs stimulated by dehydration following transfer to a hyperosmotic environment (Taylor and Grosell, 2006a) further support the suggested osmoregulatory role of intestinal anion exchange. These observations, combined with observations of elevated intestinal HCO_3^- concentrations in sturgeon exposed to hyperosmotic conditions, suggest that a role for intestinal HCO_3^- secretion in osmoregulation is perhaps an ancestral trait, which may be common to all fish (and perhaps other animals) that need to drink seawater (Taylor and Grosell, 2006a).

The substantial base output *via* the continuous release of highly alkaline rectal fluids in marine teleost fish led to the proposal by Wilson and coworkers of a role for intestinal anion exchange in acid–base balance (Wilson et al., 1996; Wilson, 1999). However, more recent studies have revealed that induced alkalosis does not stimulate intestinal base secretion (Wilson et al., 2002), and it thus appears that although base released with rectal fluids makes up a substantial exchange of basic equivalents with the environment, intestinal anion exchange does not play a role in dynamic regulation of acid–base balance. In fact, reports of total CO_2 and pH in intestinal fluids from freshwater and seawater acclimated rainbow trout, European eel and European flounder revealed that the occurrence of alkaline intestinal fluids is salinity dependent (Wilson, 1999), strongly suggesting a role in osmoregulation. The salinity dependence of intestinal anion exchange is also illustrated by total CO_2 concentrations in intestinal fluids obtained from freshwater and seawater acclimated tilapia (Fig. 2).

Examining intestinal transport of water, Na^+, Cl^- and HCO_3^- in the lemon sole using an *in situ* intestinal perfusion approach demonstrated that approximately 50% of intestinal Cl^- uptake occurred in exchange for HCO_3^- secretion while the remaining 50% was accompanied by Na^+ absorption (Grosell et al., 1999). These observations were the first to quantify the significance of intestinal anion exchange for Cl^- and thus water absorption, and highlight the anion exchange process as being quantitatively important for marine osmoregulation. Later, intestinal Cl^- and water absorption in the absence of luminal Na^+ was demonstrated using the Pacific sanddab (Grosell et al., 2001). Notably, Cl^- absorption under these conditions was matched by HCO_3^- secretion, suggesting that the Na^+ independent fraction of Cl^- absorption was accomplished by anion exchange. Furthermore, it was noted that Cl^- absorption *via* this anion exchange system must be secondary active in nature since it occurred against an electrochemical gradient even in the absence of Na^+.

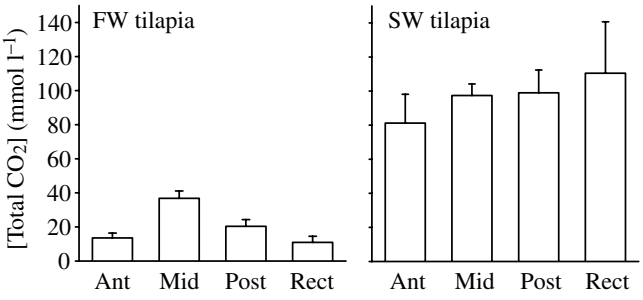

Fig. 2. Total CO_2 concentrations in fluids obtained from the anterior (Ant), mid, posterior (Post) intestine and the rectum (Rect) of freshwater (FW) and seawater (SW) acclimated *Tilapia auratus*. Values are mean ± s.e.m. *N*=10. Samples were obtained as outlined previously (Grosell et al., 1999; Grosell et al., 2001; Grosell and Jensen, 2000).

Considering the simultaneous measurements of intestinal net Na^+ and Cl^- absorption in different species of marine teleosts summarized in Table 1, it is clear that Cl^- absorption consistently exceeds Na^+ absorption regardless of species. In the first five studies listed, HCO_3^- secretion rates were measured in addition to NaCl absorption and a good correlation between the magnitude of the difference between Na^+ and Cl^- absorption and the HCO_3^- secretion is evident. This correlation strongly suggests that the excess Cl^- absorption can be attributed to Cl^-/HCO_3^- exchange. Considering all the studies in Table 1 and assuming that anion exchange can account for the excess Cl^- absorption in cases where HCO_3^- secretion was not measured, anion exchange consistently accounts for a substantial portion of Cl^- absorption, in some cases up to 71%.

Observations of Cl^- absorption in the absence of Na^+ absorption (no Na^+ on the luminal side of the intestinal epithelium) was first reported for the winter flounder (Field et al., 1978) and later for the Pacific sanddab (Grosell et al., 2001) and the European flounder (Grosell et al., 2005). In the study on the European flounder, Cl^- and water absorption was observed across flounder intestine despite lack of net Na^+ absorption even when Na^+ was present in the intestinal lumen. The salines used in the study on the European flounder were designed to mimic *in vivo* conditions characterized by a substantial uphill electrochemical gradient for Cl^- absorption. Under various experimental manipulations of Cl^- and HCO_3^- transport, a co-variation between net Cl^- uptake rates and HCO_3^- secretion rates was observed (Grosell et al., 2005). Thus, in the absence of net Na^+ uptake, the intestinal anion exchange is clearly capable of active Cl^- absorption, which in turn provides the driving force for water absorption. Furthermore, it was demonstrated that the high HCO_3^- concentrations found in intestinal fluids of marine teleost fish are the product of active HCO_3^- secretion across the intestinal epithelium mediated by Cl^-/HCO_3^- exchange. Additional components are required to account for active HCO_3^- secretion processes mediated by Cl^-/HCO_3^- exchange, which warrant a discussion of the epithelial transport mechanisms responsible for both thermodynamic uphill HCO_3^- and Cl^- transport.

Mechanisms of intestinal anion exchange

Source of substrate and energy for active transport

Active luminal secretion of HCO_3^- was recently demonstrated for European flounder (Grosell et al., 2005) and has been confirmed for gulf toadfish, which displays a Q_{10} of 1.8–3.0 for intestinal HCO_3^- secretion (Grosell and Genz, 2006). A limited number of studies have addressed the exact mechanistic nature of the intestinal anion exchange system in marine fish. These studies include pH-stat type experiments on the goby and the Japanese eel (Ando and Subramanyam, 1990; Dixon and Loretz, 1986) and more recent experiments including both pH-stat techniques and isolated intestinal sac procedures (Grosell et al., 2001; Grosell et al., 2005; Grosell and Genz, 2006; Grosell and Jensen, 1999; Wilson et al., 2002; Wilson and Grosell, 2003). From these studies, an understanding of the transport mechanisms responsible for the active HCO_3^- secretion is emerging, but numerous questions remain to be addressed and it seems that substantial species-specific differences may exist.

Apical anion exchange

Substantial evidence for an apical Cl^-/HCO_3^- exchanger has accumulated since the first documentation of HCO_3^- secretion being dependent on luminal Cl^- in the goby (Dixon and Loretz, 1986), and includes luminal Cl^- dependence in Japanese eel (Ando and Subramanyam, 1990), rainbow trout (Wilson et al., 1996), pacific sanddab (Grosell et al., 2001) and European flounder (Grosell et al., 2005). Further evidence is reduced luminal HCO_3^- secretion in the presence of DIDS in the luminal fluids of Japanese eel (Ando and Subramanyam, 1990), the European flounder (Grosell and Jensen, 1999) and the pacific sanddab (Grosell et al., 2001). Additional compelling evidence is cross-reactivity of an anion exchanger (AE1) antibody with the apical surface of the brackish water mudskipper and seawater acclimated coho salmon (Wilson et al., 2002). In agreement with intestinal HCO_3^- secretion being most pronounced in seawater *versus* freshwater acclimated fish, AE1 cross-reactivity with the apical region of coho salmon intestinal epithelium is strong in seawater acclimated individuals but absent in freshwater acclimated conspecifics (Wilson et al., 2002).

Although evidence is strong for an apical anion exchanger in marine teleost intestinal epithelium, two reports of no luminal DIDS effects on HCO_3^- secretion (Dixon and Loretz, 1986; Wilson et al., 1996) disagree with the presence of an apical anion exchanger. However, both these studies employed relatively low concentrations of DIDS and showed mucosal Cl^- dependence, still supporting the presence of an apical anion exchange process.

Source of HCO_3^-

Transepithelial HCO_3^- transport

Evidence for transepithelial HCO_3^- transport comes from observations of reduced luminal HCO_3^- secretion when serosal HCO_3^- is replaced with PO_4^{2-} in the Japanese eel (Ando and Subramanyam, 1990) and from observations of mucosal HCO_3^- secretions correlating with serosal HCO_3^- concentrations in the European flounder (Grosell et al., 2005). Furthermore, luminal HCO_3^- secretion is reduced when serosal HCO_3^- is replaced with Hepes in the gulf toadfish (Grosell and Genz, 2006). It should be noted, however, that substantial HCO_3^- secretion persists even in the absence of serosal HCO_3^- and that alternative sources of HCO_3^- appear to be able to sustain approximately 30–60% of the luminal HCO_3^- secretion at least in the goby, European flounder and gulf toadfish (Dixon and Loretz, 1986; Grosell et al., 2005; Grosell and Genz, 2006). Ando and Subramanyam, working on the Japanese eel, reported a larger contribution of serosal HCO_3^- to luminal HCO_3^- secretion but employed 24.9 mmol l^{-1} HCO_3^- in serosal salines (Ando and Subramanyam, 1990). While this HCO_3^- concentration is consistent with mammalian extracellular fluid values, it is far above the 2–10 mmol l^{-1} normally seen in

teleost fish, including eels (Marshall and Grosell, 2005), and might explain highly elevated luminal HCO_3^- secretion rates.

Nevertheless, transepithelial HCO_3^- transport requires absorption of HCO_3^- by the intestinal epithelial cells from the relatively low extracellular concentrations across the basolateral membrane, which exhibits a cytosolic negative potential difference of ~80 mV (Loretz, 1995). Luminal HCO_3^- secretion has been demonstrated to be dependent on serosal Na^+ in Japanese eel, Pacific sanddab and gulf toadfish (but apparently not in European flounder; M.G., personal observations) (Ando and Subramanyam, 1990; Grosell et al., 2005; Grosell and Genz, 2006) and is sensitive to DIDS in serosal salines in the Japanese eel (Ando and Kobayashi, 1978) and the goby (Loretz, 1995). These observations are consistent with a Na^+:HCO_3^- cotransporter (NBC) in the basolateral membrane, which would allow for HCO_3^- import against an electrochemical gradient across the basolateral membrane driven by the favorable Na^+ gradient. However, a role for NBC in intestinal HCO_3^- secretion remains to be conclusively demonstrated and it should be noted that the European flounder does not require serosal Na^+ for luminal HCO_3^- secretion (M.G., personal observations), nor is it sensitive to serosal DIDS (Grosell and Jensen, 1999), perhaps illustrating interesting species differences.

Metabolic CO_2

As mentioned above, substantial intestinal HCO_3^- secretion persists even in the absence of serosal HCO_3^-, suggesting that hydration of CO_2 within the intestinal epithelium might provide HCO_3^- for apical secretion. The source of CO_2 for hydration within the epithelial cells could be extracellular (which would require diffusion from the extracellular fluids across the basolateral membrane) or endogenous metabolic CO_2 from within the intestinal epithelium. Evidence for epithelial, endogenous metabolic CO_2 providing the substrate for HCO_3^- comes from experiments on both flounder and gulf toadfish using serosal Hepes buffered salines gassed with 100% O_2 (Grosell and Genz, 2006; Wilson and Grosell, 2003). Under these conditions, 60–80% of control HCO_3^- secretion rates (physiological HCO_3^- concentrations and partial pressure of CO_2 in serosal fluids) persists, showing a significant contribution from endogenous epithelial metabolic CO_2. In addition, it appears that extracellular CO_2 may provide the substrate for cellular CO_2 hydration and thus HCO_3^- secretion at least in the European flounder. Experiments with elevated (2% compared to controls of 0.5%) serosal CO_2 levels revealed increased luminal HCO_3^- secretion as well as elevated net Cl^- and water absorption (Grosell et al., 2005). It should be noted, however, that reduction of extracellular CO_2 concentration did not result in reduced HCO_3^- secretion, suggesting that while extracellular super-physiological CO_2 levels may contribute to elevated luminal HCO_3^- secretion, other sources of HCO_3^- are sufficient to sustain basal control levels of HCO_3^- secretion.

Accepting that a substantial part of the secreted HCO_3^- is derived from endogenous metabolic CO_2 allows for predictions of metabolic rates of marine teleost intestinal epithelia. Such considerations led to an estimate of intestinal epithelium metabolic rate being at least five- to eightfold higher than corresponding mass-specific whole animal consumption rates (Grosell et al., 2001), which is supported by the high mitochondrial density in marine teleost intestinal epithelia (Walsh et al., 1991). This five- to eightfold higher mass-specific metabolic rate is estimated assuming that all metabolic CO_2 produced translates to HCO_3^- secretion. However, it is likely that some CO_2 diffuses from the epithelial cells to the extracellular fluid, perhaps indicating that the metabolic rate may be even higher than five- to eightfold that of mass-specific whole animal rates. In addition to the osmoregulatory role of the intestine, other functions including digestion, nutrient absorption, endocrine activity and barrier functions (Mommsen et al., 2003) are conceivably energetically costly and may explain the need for high abundance of mitochondria. The hydration of the metabolic waste product, CO_2, and the subsequent exchange of HCO_3^- for Cl^- effectively exchanges a gas that exerts limited osmotic pressure with a main osmolyte, Cl^-, which in turn provides the osmotic driving force for cellular water uptake.

Three studies have demonstrated the importance of the enzyme carbonic anhydrase for intestinal HCO_3^- secretion in the goby, the rainbow trout and the gulf toadfish by use of pharmacological inhibitors (Dixon and Loretz, 1986; Grosell and Genz, 2006; Wilson et al., 1996). However, although carbonic anhydrase mediated CO_2 hydration contributes to overall CO_2 hydration within the intestinal epithelium, it should be noted that relatively low inhibition (30–40%) of HCO_3^- secretion was observed for both species, despite the use of relatively high inhibitor concentrations. These observations may suggest that non-mediated CO_2 hydration can account for some of the overall basal HCO_3^- production and thus excretion, but may also indicate that experimental conditions prevented full carbonic anhydrase inhibition.

Basolateral H^+ extrusion

Regardless of the CO_2 source (extracellular *versus* endogenous), cellular CO_2 hydration produces H^+ in addition to HCO_3^-, and the H^+ must be excreted from the epithelial cells to prevent reversal of the hydration reaction and thereby allow for accumulation of cellular HCO_3^- for anion exchange. Furthermore, it seems clear that the H^+ extrusion must occur across the basolateral membrane since the intestinal epithelium exhibits substantial net base secretion (serosal → mucosal) (Grosell et al., 2001; Grosell et al., 2005).

A prediction of similar basolateral H^+ extrusion rates and apical HCO_3^- secretion rates in the gulf toadfish intestine was confirmed using pH-stat titration techniques in both mucosal and serosal salines, conclusively demonstrating that H^+ ions arising from CO_2 hydration are extruded across the basolateral membrane (Grosell and Genz, 2006). The importance of this basolateral H^+ extrusion for apical Cl^-/HCO_3^- exchange is clearly illustrated in the gulf toadfish by reversible, H^+ concentration dependent inhibition of luminal HCO_3^- secretion when serosal pH is reduced from 7.8 to 7.4, 7.0 and 6.6

(Grosell and Genz, 2006). Reduction of serosal pH would make H^+ gradients across the basolateral membrane less favorable for H^+ extrusion, which presumably results in reduced cellular HCO_3^- for apical anion exchange.

Basolateral H^+ extrusion seems to occur *via* a Na^+/H^+ exchange (NHE) mechanism, at least in the gulf toadfish, as luminal HCO_3^- secretion relies on serosal Na^+ even in the absence of serosal HCO_3^- (Grosell and Genz, 2006). Although HCO_3^- secretion is insensitive to even high concentrations of amiloride and EIPA [5-(*N*-ethyl-*N*-isopropyl) amiloride] added to the serosal medium, dependence on Na^+ gradients is clearly demonstrated by experiments with the NKA inhibitor ouabain. When NKA is inhibited and Na^+ gradients are partly depleted, luminal HCO_3^- secretion is greatly reduced, presumably because H^+ extrusion *via* an NHE-like mechanism is reduced (Grosell and Genz, 2006). These recent observations of ouabain sensitivity confirm similar observations on the goby (Dixon and Loretz, 1986) and observations of luminal HCO_3^- secretion being dependent on serosal Na^+ in the Japanese eel (Ando and Subramanyam, 1990). However, it should be noted that in both the studies on the goby and the eel, serosal salines contained HCO_3^- and that the apparent dependence on Na^+ gradients could also be explained by basolateral HCO_3^- import *via* NBC.

However, for the gulf toadfish where an NHE-like transporter is critical for luminal HCO_3^- secretion, and also for goby and eel where NBC or NHE appear to play a role in HCO_3^- secretion, Na^+ gradients are important. Thus, HCO_3^- secretion, at least in gulf toadfish, goby and eel, rely on electrochemical gradients established by NKA, which ultimately fuel basolateral H^+ extrusion and thereby the secondary active HCO_3^- and Cl^- transport by the apical anion exchanger.

An apparent lack of serosal Na^+ dependence in the European flounder (M.G., personal observation) may imply that species differences in transport mechanisms exist. An alternative mechanism might include a basolateral H^+-pump that could fuel basolateral H^+ extrusion and thereby the active HCO_3^- secretion across the apical membrane independently of Na^+ gradients.

Alkaline precipitation

In addition to the more direct role for anion exchange in Cl^- and thereby water absorption, an indirect but possibly quantitatively important consequence of highly alkaline intestinal fluids is the precipitation of Ca^{2+} and Mg^{2+} carbonates within the intestinal lumen. The presence of macroscopic light colored solids in the intestinal lumen even of starved fish had already been noted by Smith in his classic study published in 1930 (Smith, 1930) and has since been noted to be most pronounced at higher salinities (Shehadeh and Gordon, 1969). Walsh and coworkers were the first to report that these intestinal solids consisted primarily of Mg^{2+} and Ca^{2+} carbonates (Walsh et al., 1991) but the overall importance of this precipitation within the intestinal lumen has only recently been recognized.

Carbonate precipitates account for ~20% of rectal base excretion in marine fish under control conditions (Wilson et al., 1996; Wilson et al., 2002; Wilson and Grosell, 2003), whereas 30–65% of the rectal Ca^{2+} excretion can be accounted for by the precipitates (Shehadeh and Gordon, 1969; Wilson and Grosell, 2003). Considering that only a modest fraction of Ca^{2+} ingested with seawater is absorbed across the intestinal epithelium and that as much as 85% of the ingested seawater is absorbed, intestinal fluid Ca^{2+} concentrations could be expected to be approximately sixfold higher than corresponding seawater Ca^{2+} concentrations (~10 mmol l^{-1}). However, numerous studies report intestinal fluid Ca^{2+} concentrations at 5 mmol l^{-1} (Marshall and Grosell, 2005), which in earlier reports was erroneously interpreted to be the result of substantial intestinal Ca^{2+} uptake (Evans, 1993; Hickman, 1968; Karnaky, 1998). The formation of carbonate precipitates, which is a direct consequence of the high HCO_3^- concentration and alkaline conditions, account for the majority of the Ca^{2+} lost from the intestinal fluids and has important consequences for the osmotic pressure of the intestinal fluid. An estimated reduction of ~70 mOsm resulting from the precipitation of Ca^{2+} and CO_3^{2-} in the intestinal fluids (Wilson et al., 2002) is an obvious benefit for intestinal fluid absorption and has also been demonstrated to be important for Ca^{2+} homeostasis (Wilson and Grosell, 2003).

In vitro versus *in vivo* discrepancies

Thermodynamic considerations

In vitro studies performed on isolated intestinal epithelia display Cl^- uptake rates that are substantially higher than corresponding Na^+ uptake rates (Table 1), and it appears that all regions of the intestine, at least in teleost fish, display comparable HCO_3^- secretion rates *in vitro* (Wilson et al., 2002). These two observations appear to be in contrast to the situation *in vivo*, where intestinal fluid Cl^- concentrations always exceed Na^+ concentrations and where a substantial increase of HCO_3^- concentrations in intestinal fluids is seen in the anterior region with little or no further increase along the gastro-intestinal tract. These two discrepancies between *in vitro* and *in vivo* observations will be discussed below.

Considering first the issue of Cl^- *versus* Na^+ uptake rates, the apparent discrepancy between *in vitro* and *in vivo* observations can be attributed to different concentrations of Cl^- and Na^+ in seawater. In general, Cl^- concentrations exceed Na^+ concentrations in seawater by ~70 mmol l^{-1}, which means that seawater ingestion results in higher gastrointestinal intake of Cl^- than Na^+. Further, desalinization in the esophagus occurs *via* both passive and active equimolar Na^+ and Cl^- absorption (Kirsch and Meister, 1982; Parmelee and Renfro, 1983; Smith, 1930; Wilson et al., 1996). With little or no transport across the gastric mucosa in starved fish, the consequence of the higher concentrations of Cl^- than Na^+ in seawater and equal molar Na^+ and Cl^- absorption in the esophagus is that fluids entering the intestine contain higher concentrations of Cl^- than Na^+. However, while intestinal fluid Cl^- concentrations remain

higher than the corresponding Na^+ concentrations as fluids move along the intestine, the difference between Na^+ and Cl^- concentrations is reduced and can be as low as 10–30 mmol l^{-1} (Grosell et al., 2001; Marshall and Grosell, 2005; Taylor and Grosell, 2006a). The reduced difference between Na^+ and Cl^- concentrations in intestinal fluids compared to seawater must be the result of intestinal Cl^- absorption in excess of Na^+ absorption. Using the gulf toadfish as an example, gastro-intestinal seawater intake and processing is illustrated in Fig. 3A,B. Intake of Na^+ and Cl^- with seawater (Fig. 3A) was calculated assuming a drinking rate of 2 ml kg^{-1} h^{-1} and seawater concentrations of 489 and 420 mmol l^{-1} Cl^- and Na^+, respectively. The amount of Na^+ and Cl^- passing the esophagus is estimated from Na^+ and Cl^- concentrations measured in stomach fluids of starved fish (Marshall and Grosell, 2005) and an assumption of no esophageal water absorption. The amount of Na^+ and Cl^- present in various segments of the intestine (Fig. 3A) is estimated from Na^+ and Cl^- concentrations measured in toadfish intestinal fluids (Taylor and Grosell, 2006a) and an assumption of 20% fluid absorption in each of the four intestinal segments, yielding a total fractional fluid absorption of 80% (Marshall and Grosell, 2005). These simple considerations reveal that the majority of the ingested Na^+ and Cl^- are absorbed in the esophagus and the anterior intestine. From differences in concentrations of Na^+ and Cl^- among various gastro-intestinal segments, regional uptake rates of Na^+ and Cl^- can be estimated as illustrated in Fig. 3B. From this illustration it becomes apparent that esophageal salt absorption also appears to be equimolar with respect to Na^+ and Cl^- *in vivo*, but that the same is not the case for the intestine. In all segments of the toadfish intestine, *in vivo* absorption of Cl^- exceeds the corresponding Na^+ absorption, with the anterior intestine displaying the largest absolute difference between Na^+ and Cl^- absorption. In this example (Fig. 3A,B), based on toadfish, Cl^- absorption exceeds Na^+ absorption by 12–57%, which is generally lower than values for isolated or perfused intestinal epithelia (Table 1) where Cl^- absorption can be up to 71% higher than Na^+ transport. Although the relative contribution of excess Cl^- absorption appears to be lower *in vivo* than *in vitro* or *in situ*, it is still of quantitative significance especially in the anterior region of the intestine. In agreement with the argument presented above that Cl^-/HCO_3^- exchange accounts for this excess Cl^- absorption, both Cl^- absorption (Fig. 3B) and intestinal HCO_3^- secretion (Fig. 4) are most pronounced in the anterior segment of the intestine *in vivo*.

The second discrepancy between *in vivo* and *in vitro* observations is the relatively silent *in vivo* Cl^-/HCO_3^- exchange activity in distal segments of the intestine despite the obvious capacity for HCO_3^- secretion in these segments *in vitro*. As illustrated in Fig. 4, all intestinal segments, when isolated from the gulf toadfish, display high HCO_3^- secretion rates, which is in agreement with observations from European flounder, Pacific sanddab and lemon sole (Grosell et al., 1999; Grosell et al., 2001; Grosell and Jensen, 1999). The high secretion rates in the distal segments of the intestine are in contrast to the limited or lack of an increase in luminal HCO_3^-

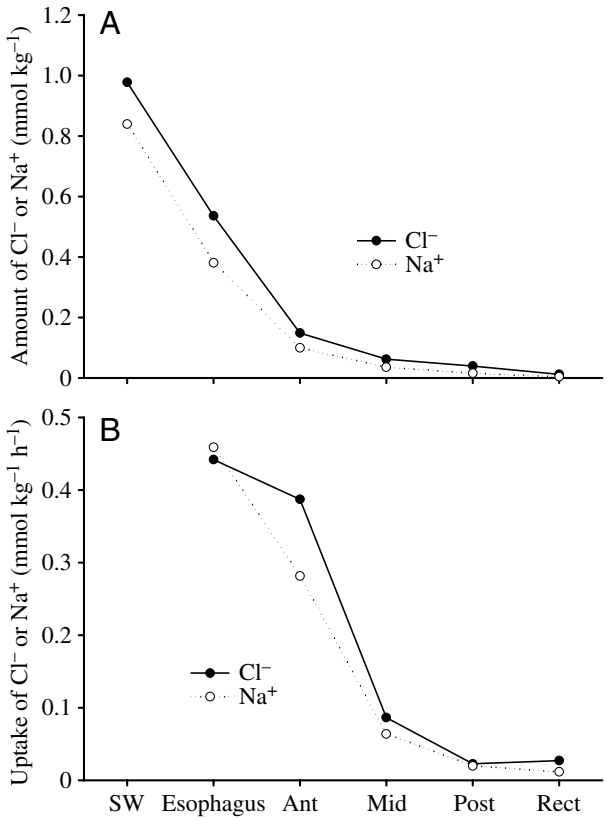

Fig. 3. (A) The amount of Na^+ and Cl^- ingested by a marine teleost fish assuming a drinking rate of 2 ml $kg^{-1}h^{-1}$ (Marshall and Grosell, 2005) and the amount of Na^+ and Cl^- present in fluids passing through the esophagus, the anterior (Ant), the mid, the posterior (Post) and the rectal segment (Rect) of the intestine. The amount of Na^+ and Cl^- passing through the esophagus was calculated from concentrations of Na^+ and Cl^- in stomach fluids from starved fish (Kirsch and Meister, 1982; Parmelee and Renfro, 1983; Smith, 1930; Wilson et al., 1996), assuming that no water absorption occur across the esophagus (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983). Amounts of Na^+ and Cl^- present in the intestinal segments were calculated from concentrations of Na^+ and Cl^- found in unfed toadfish intestinal fluids (Taylor and Grosell, 2006a) and a fractional water absorption of 20% in each intestinal segment, yielding a total fractional water absorption of 80% (Marshall and Grosell, 2005). Note that the concentrations of Cl^- exceed corresponding Na^+ concentrations in all gastro-intestinal segments but that the absolute concentration difference between the two ions diminishes as fluids are passing along the intestine. (B) Net Na^+ and Cl^- uptake rates (mmol kg^{-1} h^{-1}) occurring across different segments of the gastro-intestinal tract calculated from the different amounts of Na^+ and Cl^- presented in A. Note the equal molar Na^+ and Cl^- absorption in the esophagus and the substantial excess Cl^- absorption in the anterior intestine. See text for further details.

concentrations *in vivo* as fluids are moving along the intestine of all four species. This discrepancy is likely related to the *in vitro* conditions employed for measurement of HCO_3^- transport rates, which include the use of HCO_3^- free luminal salines of identical composition in all intestinal segments. The reduction in HCO_3^- concentrations in luminal salines is

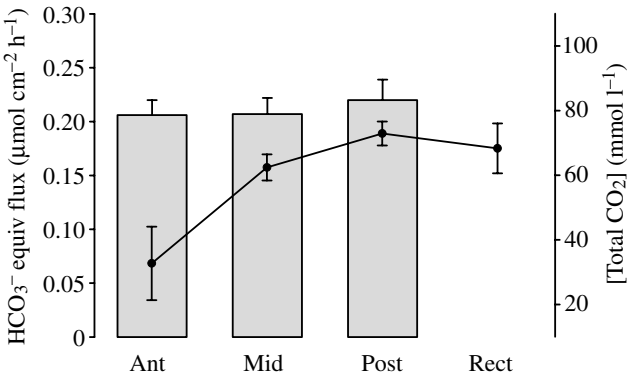

Fig. 4. Intestinal HCO_3^- secretion rates (bars, means + s.e.m., N=8) of isolated anterior (Ant), mid and posterior (Post) segments of toadfish intestine and total CO_2 concentrations of luminal fluids (circles; means ± s.e.m., N=8) obtained from the anterior, mid, posterior and rectal portion (Rect) of the toadfish intestine. Flux rates in isolated intestinal segments were measured using the same luminal saline (identical HCO_3^- and Cl^- concentrations for all segments) and serosal saline for all three segments (Grosell and Genz, 2006). Total CO_2 values are from a recent study on unfed toadfish (Taylor and Grosell, 2006a).

necessary to avoid $CaCO_3$ precipitation but is obviously different from *in vivo* situations where HCO_3^- builds up to high concentrations, especially in the more distal segments. Further, *in vitro* measurements to date have used the same Cl^- concentrations for anterior, mid and posterior intestinal segments, which again contrast with the *in vivo* situation where Cl^- concentrations in intestinal fluids generally become lower in more distal segments of the intestine. The likely explanation for the low HCO_3^- secretion rates in distal intestinal segments *in vivo*, despite the obvious HCO_3^- transport capacity seen *in vitro*, is that Cl^- and HCO_3^- gradients become less favorable for the anion exchange process as fluids are moving through, and are being processed by the intestine.

This argument leads to examination of the thermodynamics of intestinal anion exchange to assess the potential contribution of this transport process to active Cl^- absorption in different intestinal segments *in vivo*. The Nernst equation (below) allows for calculation of the equilibrium potentials (V_{eq}, in V) associated with ion gradients across cell membranes and thereby to evaluate if ionic gradients display equilibrium or are the product of active transport:

$$V_{eq} = [(RT)/(Fz)]\cdot 2.303\cdot\log([X_o]/[X_i]) ,$$

where $[X_o]$ and $[X_i]$ refer to the concentrations of the ion in question outside and inside the cell respectively and R,T,F,z have their usual meaning. Note that the natural logarithm is replaced with the 10-based logarithm by applying 2.303 as correction factor.

The electrochemical potential (V_{ec}, in V) acting on the ion in question is simply the difference between the relevant membrane potential (V_m) and V_{eq}.

Considering Cl^- distribution across the apical membrane in the anterior intestine with a luminal Cl^- concentration of 93 mmol l^{-1} (Taylor and Grosell, 2006a), an intracellular Cl^- concentration of 30 mmol l^{-1} (Duffey, 1979; Stewart et al., 1980) and an apical V_m of –100 mV (Loretz, 1995), V_{ec} for Cl^- is –71 mV at room temperature. Thus, cellular Cl^- is far above thermodynamic equilibrium across the apical membrane and apical Cl^- absorption must occur *via* (secondary) active transport.

The feasibility of the combined movement of two or more ions *via* the same transport protein can be evaluated by considering the sum of V_{ec} values for the ions in question. Thus apical equimolar anion exchange, transport in the direction of Cl^- absorption and HCO_3^- secretion, can occur when $V_{ec}(Cl^-)+[-V_{ec}(HCO_3^-)]>0$. Note that the contribution from $V_{ec}(HCO_3^-)$ is negative since transport is in the opposite direction to Cl^-.

At present, there are no measurements of cytosolic HCO_3^- concentrations in intestinal epithelial cells but all other components of the equation are available (V_m, luminal Cl^- and HCO_3^- and cytosolic Cl^- concentration). With this information and the observation that HCO_3^- secretion occurs *in vivo* in the anterior and to some extent in the middle segment of the toadfish intestine, cytosolic concentrations of HCO_3^- required for activity of apical anion exchange [V_{ec}(anion exchange)>0] can be estimated. Calculated electrochemical potentials for apical anion exchange determined for cytosolic HCO_3^- concentrations ranging from 1.5 to 40 mmol l^{-1} are presented in Fig. 5. From these calculations it is obvious that the cytosolic HCO_3^- concentration must be >10 mmol l^{-1} for anion exchange to occur in the gulf toadfish *in vivo* in the anterior region of the intestine and ~40 mmol l^{-1} for anion exchange to occur in the mid region.

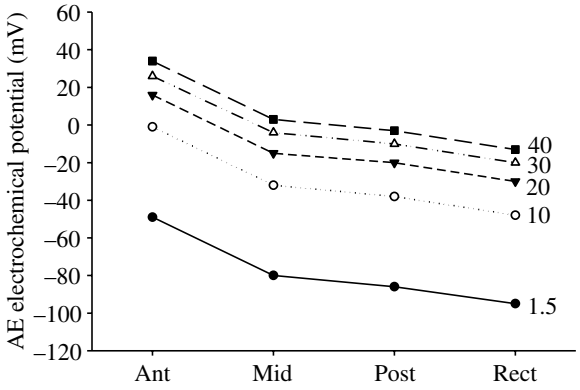

Fig. 5. Electrochemical potential for the apical anion exchange (AE) process calculated assuming intracellular Cl^- concentration and apical membrane potential of 30 mmol l^{-1} and –100 mV, respectively, and using luminal Cl^- and HCO_3^- concentrations from unfed gulf toadfish (Taylor and Grosell, 2006a) and different intracellular HCO_3^- concentrations ranging from 1.5 to 40 mmol l^{-1} (indicated by different symbols). Absorption of Cl^- and HCO_3^- secretion *via* 1:1 anion exchange can only occur when the AE electrochemical potential is >0, which requires intracellular HCO_3^- concentrations of >10 mmol l^{-1} in the anterior intestine and close to 40 mmol l^{-1} in the mid intestine. See text for further details.

These values agree fairly well with the estimated cytosolic HCO_3^- concentration of >9.1 mmol l^{-1} required to sustain HCO_3^- secretion in the Pacific sanddab (Grosell et al., 2001). The calculations presented in Fig. 5 are based on the assumption that cytosolic Cl^- and HCO_3^- concentrations remain constant along the intestine and demonstrate why there is no further increase in luminal HCO_3^- concentrations from the anterior and mid segments to the more distal segments. At any given cytosolic HCO_3^- concentration, the gradual depletion of luminal Cl^- concentrations, combined with the build up of HCO_3^- concentrations in the more distal segments, shifts V_{ec}(anion exchange) to being less favorable for Cl^- absorption and HCO_3^- secretion. The experimental conditions used for *in vitro* measurements are different in having low HCO_3^- concentrations and identical Cl^- concentrations in the lumen of all intestinal segments. These *in vitro* conditions impose fewer thermodynamical constraints on apical anion exchange than is the case *in vivo*, especially for the distal segments of the intestine, and explain why distal segments display high HCO_3^- secretion rates *in vitro* but not *in vivo*.

Cytosolic HCO_3^- concentrations of 10–40 mmol l^{-1} seem high compared to the 1.5 mmol l^{-1} HCO_3^- estimated from the assumed intracellular pH of 7.4, P_{CO_2} of 2.3 mmHg (307 Pa) and no CO_2 diffusion limitation (Grosell et al., 2001). However, based on trout white muscle, which displays 4–5 times higher P_{CO_2} (Wang, 1998), it seems reasonable to assume that intracellular P_{CO_2} might be also be substantially higher in the intestinal epithelium and that this might result in higher cytosolic HCO_3^- concentrations. In addition, production of endogenous CO_2 may occur at high rates in this metabolically active tissue, acting to further increase P_{CO_2} and thus HCO_3^- concentrations. Based on these considerations alone, cytosolic HCO_3^- concentrations of ~ 10 mmol l^{-1} may not seem unrealistic.

Local, high HCO_3^- concentrations in micro-environments in close proximity of the apical anion exchanger may explain Cl^-/HCO_3^- exchange under conditions where $\geqslant$10 mmol l^{-1} cytosolic HCO_3^- is required and could be the result of recently discovered physical interactions between carbonic anhydrase II (CAII) and members of the anion exchanger family (AE1, AE2, AE3 and DRA) (Sterling et al., 2001; Sterling et al., 2002). Binding of functional CAII to the COOH terminus of AE1, AE2, AE3 and DRA has been demonstrated by coimmunoprecipitation and is required for maximal HCO_3^- transport activity in mammals (Sterling et al., 2001; Sterling et al., 2002). The direct interaction between AE and CAII, effectively forming a metabolon, is believed to accelerate HCO_3^- production and transport by minimizing the diffusional distance between CAII and the anion exchanger (Sterling et al., 2002), and has been reported to stimulate CAII activity directly (Scozzafava and Supuran, 2002).

Absorbate composition

Hyperosmotic?

The high luminal HCO_3^- secretion rates have implications for the composition of the fluid absorbed by the intestine of marine fish. Multiple investigations reporting simultaneous net Na^+, Cl^- and fluid absorption rates allow for calculation of Na^+ and Cl^- concentrations and osmotic pressure in absorbed fluids (Table 2). The osmotic pressure of absorbed fluid as presented in Table 2 could not be calculated as just the sum of Na^+ and Cl^- concentrations because considerable differences exist between the concentration of these two major electrolytes in the absorbed fluids. Without exception, calculated Cl^- concentrations exceed Na^+ concentrations in absorbed fluids (whenever both ions were measured), which is a simple consequence of higher intestinal net Cl^- and than Na^+ uptake rates as also demonstrated in Table 1. Since charge balance must exist in the absorbed fluid, cation(s) other than Na^+ must be absorbed in conjunction with Na^+ and Cl^- or substantial transepithelial secretion of anions must occur. While HCO_3^- secretion may contribute to charge balance it should be noted that a substantial fraction of the secreted HCO_3^- is derived from hydration of cellular CO_2 (see above) and that the result of the anion exchange therefore represents a net transepithelial gain of anionic electrolytes.

Assuming no anion secretion, the 'missing' cation(s) in the absorbed fluid must be equal in charge to the difference between the calculated concentrations of Na^+ and Cl^-. The calculation of osmotic pressure in fluids absorbed (Table 2) by marine fish intestines took the concentration of the missing cation(s) into account, and assumed mono-valence and an osmotic coefficient of 1 for solutes in the absorbate. Under these assumptions, with a single exception (*Fundulus heteroclitus*), absorbate is highly hyperosmotic with respect to both luminal and the extracellular fluids, which is in marked contrast to the iso-osmotic fluid absorbed by other vertebrate epithelia, the gall bladder, the renal proximal tubule and the small intestine in tetrapods (Larsen, 2000; Larsen et al., 2002; Nedergaard et al., 1999).

Admittedly, the osmotic coefficient of NaCl is less than 1 and would be even lower if the absorbed fluids were dominated by $MgSO_4$ (0.91 and 0.56, respectively) (Taylor and Grosell, 2006b). However, considering that NaCl likely is the dominating osmolyte in fluids absorbed by the intestine and a conservative osmotic coefficient of 0.8, the calculated osmotic pressure of fluids absorbed by a range of marine fish (Table 2) still appears to be hyperosmotic. If the difference between Cl^- and Na^+ absorption is accounted for by absorption of divalent rather than a monovalent cations, the predicted osmotic pressure would be reduced because a lower molar concentration would be required to offset the apparent charge imbalance. Similarly, considering that part of the difference between Cl^- and Na^+ absorption could be accounted for by transepithelial HCO_3^- secretion, the true anion–cation Gab would be less than assumed and a 'missing cation' would thus contribute less to the osmotic pressure of absorbed fluids.

While the result of these three factors (reduced osmotic coefficient, absorption of divalent cations and transepithelial HCO_3^- secretion) may reduce the estimated osmotic pressure listed in Table 2, absorbate still appears hypertonic in most cases. This is clear in at least 6 of 10 cases in which Na^+

Table 2. *Net uptake rates of Cl^-, Na^+ and water, and calculated concentrations of Na^+ and Cl^- and concentrations of unknown cations required for charge balance in fluid absorbed by the intestinal epithelium of different marine or euryhaline fish*

Species	Net flux (μl cm^{-2} h^{-1}, ml kg^{-1} h^{-1} or μl g^{-1} h^{-1})			[Na^+] in absorbed fluid (mmol l^{-1})	[Cl^-] in absorbed fluid (mmol l^{-1})	[Missing cation] (mmol l^{-1})	Osmotic pressure in absorbed fluid (mOsm)
	H_2O	Na^+	Cl^-				
Platichthys flesus[1,†]	2.1	–0.37	0.58	–	274	274	548
Citharichthys sordidus[2,*]	14.0	2.3	3.5	164	250	86	500
Parophrys vetulus[3,*]	4.4	–	1.5	–	341	–	682
Parophrys vetulus[3,§]	2	0.2	0.4	100	200	100	400
Anguilla anguilla[4,§] (FW)	–	–	–	199	262	63	651
Anguilla anguilla[4,§] (SW)	–	–	–	238	280	42	644
Anguilla anguilla[4,§] (⅓SW)	–	–	–	261	291	30	642
Salmo gairdneri[5,#] (⅓SW)	1.1	0.17	0.23	155	209	54	418
Salmo gairdneri[5,#] (½SW)	3.0	0.79	0.99	263	330	67	660
Salmo gairdneri[5,#] (SW)	4.3	2.33	2.82	542	656	114	1312
Fundulus heteroclitus[6,‡] (Posterior, resting)	8.4	–	2.5	–	136		272
Fundulus heteroclitus[6,‡] (Posterior, stimulated)	–7.4	–	–3.4	–	460		920 (secretion)
Paralichthys lethostigma[7,#]	3.5	1.9	2.1	543	600	57	1200
Lampetra fluviatilis[8,‡] (Anterior intestine)	39	4.49	9.32	115	239	124	478

FW, freshwater; SW, seawater.

Predicted osmotic pressure of the absorbed fluid was calculated assuming an osmotic coefficient of 1.

[†]Transport by isolated epithelia under asymmetrical conditions in absence of an osmotic gradient.

*Transport by isolated epithelia under asymmetrical conditions with osmotic pressure in serosal fluids>mucosal fluids.

[‡]Transport by isolated epithelia under symmetrical conditions.

[§]*In situ* perfusion.

#Based on drinking rate and intestinal content.

1 (Grosell et al., 2005); 2 (Grosell et al., 2001); 3 (Grosell et al., 1999); 4 osmotic pressure calculated from reported NaCl concentration in absorbate and cation:anion Gab assuming charge balance in absorbate (Skadhauge, 1974); 5 fish were allowed to drink the exposure medium (Shehadeh and Gordon, 1969); 6 (Marshall et al., 2002); 7 fish were allowed to drink the exposure medium (Hickman, 1968); 8 (Pickering and Morris, 1973).

concentrations were calculated to be substantially higher than 150 mmol l^{-1}, which is typical of iso-osmotic fluids transported by other vertebrate epithelia.

The reason for this difference between the marine teleost intestine and other vertebrate leaky epithelia involved in water absorption is unknown but may be related to the unique challenges faced by the marine fish intestinal epithelium.

In terrestrial vertebrates, the gall bladder and the renal proximal tubules *in vivo* are exposed to solutions of similar composition on the luminal and serosal epithelial surfaces, although this is not necessarily the case for the intestine of these organisms. Intestinal fluids in terrestrial vertebrates are dominated by monovalent electrolytes, like Na^+, Cl^- and K^+ and, to some extent, HCO_3^- (Karasov and Hume, 1997). In contrast to these epithelia, the marine intestine is exposed to a rather unique luminal environment dominated by divalent ions, especially Mg^{2+} and SO_4^{2-}, which are effectively excluded by the intestinal epithelium. Marine fish are in excess of both Mg^{2+} and SO_4^{2-} and the main homeostatic control of these divalent electrolytes appears to be renal secretion (Marshall and Grosell, 2005). However, only a modest fraction of Mg^{2+} and SO_4^{2-} ingested with seawater is absorbed *via* the intestine (Marshall and Grosell, 2005). This relatively low uptake of Mg^{2+} and SO_4^{2-} is remarkable considering the up to >100-fold concentration difference across the intestinal epithelium of both ions and the substantial fluid absorption, and likelihood of solvent drag, displayed by this organ. Thus, in contrast to other epithelia displaying iso-osmotic fluid absorption, the marine fish intestine absorbs a NaCl rich solution from a solution strongly dominated by Mg^{2+} and SO_4^{2-}. Whether high luminal concentrations of divalent ions necessitate absorption of hyper-osmotic fluid remains to be tested.

Missing cationic charge

While it appears that fluids absorbed by the marine fish intestine likely are hyper-osmotic, the missing cation(s) required for charge balance remains to be conclusively identified. The quantitative importance of this illusive ion is illustrated by calculated concentration differences between Na^+ and Cl^- in the absorbed fluids ranging from 30–274 mmol l^{-1}. No study to date has evaluated the simultaneous net transport across the intestinal epithelium of water and all electrolytes present in luminal and serosal fluids, preventing a firm conclusion about the identity of the ions present in absorbed fluids to be drawn. However, the available reports appear to provide some insight into this question.

One obvious thought is that K^+, which is absorbed *via* NKCC (see above), accounts for (some of) the missing cationic charge in the absorbed fluids. However, considering that seawater K^+ concentrations are ~10 mmol l^{-1} and assuming this is also the case for fluids moving from the stomach into the intestine, it is clear that K^+ alone cannot account for the high concentrations of cationic charge missing from the absorbed fluids. Assuming a 2 ml kg^{-1} h^{-1} drinking rate and seawater K^+ concentrations, an 80% fractional fluid absorption and a K^+ concentration of 1 mmol l^{-1} in the rectal fluids (Marshall and Grosell, 2005; Taylor and Grosell, 2006a), the K^+ concentration in absorbed fluids can be calculated to be: (0.002 l 10 mmol l^{-1} K^+–0.0004 l 1 mmol l^{-1} K^+)/0.016 l=12 mmol l^{-1}. This value falls short of accounting for the concentrations of cationic charge presented in Table 2. Similar considerations based on information in table 6.3 in a recent review (Marshall and Grosell, 2005) reveals that the concentrations of Mg^{2+} and Ca^{2+} in absorbed fluids are 12.5 and 2.5 mmol l^{-1}, respectively. Valence considered, K^+, Mg^{2+} and Ca^{2+} combined may thus account for a total of 42 mEquiv cationic charge, which seems sufficient to offset the charge imbalance in a few of the cases summarized in Table 2. However, it should be recognized that some SO_4^{2-} (25 mmol l^{-1} or 50 mEquiv anionic charge (Marshall and Grosell, 2005) is likely to be present in the absorbed fluids, which would effectively cancel out the contribution by K^+, Mg^{2+} and Ca^{2+}. It should also be noted that the combined cationic charge accounted for by K^+, Mg^{2+} and Ca^{2+} falls far short of accounting for the charge imbalance seen in most cases (Table 2).

A more likely, but perhaps surprising, cation accounting for the apparent imbalance is H^+ arising from the CO_2 hydration fueling the apical anion exchange process. Arguments have previously been made to suggest that basolateral H^+ secretion from intestinal epithelial cells is necessary to sustain the substantial net base secretion displayed by the marine fish intestine (Grosell et al., 2001; Grosell et al., 2005). Most recently, it was demonstrated experimentally that significant basolateral H^+ secretion occurred in the gulf toadfish intestine and that this secretion was necessary for luminal HCO_3^- secretion (Grosell and Genz, 2006). Measured basolateral H^+ secretion rates and water absorption in the anterior intestine were found to be 0.30±0.02 μmol cm^{-2} h^{-1} and 0.92 μl cm^{-2} h^{-1}, respectively, equating to an H^+ concentration of 326 mmol l^{-1} in the absorbed fluids (Grosell and Genz, 2006). Such H^+ concentration easily accounts for the missing cationic charge illustrated in Table 2 and strongly suggests simultaneous absorption of water and protons by the intestinal epithelium. The derived and theoretical pH of the intestinal absorbate in toadfish is highly acidic (pH<1), which may be a common feature for most marine fish. Absorption of such acidic fluids would appear to pose a challenge for the paracellular space and the interstitium, but fluids contained in these compartments will buffer the acidic absorbate at least to some extent and it is unlikely that such extremely low pH values can actually be found in the paracellular space or the interstitium.

It is worth noting that the measurements of serosal H^+ secretion in the gulf toadfish were performed using pH-stat techniques, which arguably favor apical HCO_3^- secretion and therefore basolateral H^+ excretion. This in turn may result in an overestimation of H^+ concentrations in fluids absorbed by the intestine under *in vivo*-like conditions. However, similar conclusions of a likely acidic absorbate were derived from measurements using gut sacs in which HCO_3^- was allowed to accumulate in the lumen during measurements (Grosell et al., 2005).

While intestinal HCO_3^- secretion is not directly involved in

dynamic acid–base balance, adjustment of acidic fluid absorption and transepithelial HCO_3^- transport from the extracellular fluids to the intestinal lumen, in response to changing environmental salinity, must have consequences for systemic acid–base balance. Indeed, experimentally induced elevations in intestinal HCO_3^- secretion *in vivo* result in elevated branchial secretion of acid-equivalents and reduction of plasma total CO_2 levels in the European flounder (Wilson and Grosell, 2003).

Conclusions

Apical anion exchange contributes to osmoregulation, at least in marine teleost fish. Recent reports of intestinal HCO_3^- secretion in sturgeon and bamboo shark, which drink when challenged with hyperosmotic conditions, confirm the role of the AE exchange system in osmoregulation and suggest that this phenomenon is perhaps more widespread than previously recognized (Taylor and Grosell, 2006a; Wilson, 1999; Wilson et al., 2002). Apical anion exchange explains high luminal HCO_3^- concentrations and alkaline conditions in the marine teleost intestine and is responsible for a considerable portion of intestinal Cl^- uptake. Hydration of endogenous metabolic CO_2, partly mediated by CA, provides HCO_3^- as cellular substrate for the anion exchange process by effectively exchanging the metabolic waste product (CO_2) for uptake of an osmolyte, which allows for cellular water uptake. Basolateral extrusion of H^+ arising from the hydration reaction is critical for luminal HCO_3^- secretion and the basolateral NKA provides energy for both secondary active uptake of Cl^- and the secretion of HCO_3^- by establishing conditions favorable for basolateral Na^+:H^+ exchange, presumably *via* a NHE-like protein. Transepithelial HCO_3^- transport contributes to intestinal HCO_3^- secretion and may be mediated by a basolateral NBC-like carrier. Thermodynamic considerations predict high cellular HCO_3^- concentrations in the intestinal epithelium, which may in part be accounted for by high metabolic activity and by a close association of AE and CA.

Although all intestinal segments when isolated exhibit the capacity for anion exchange it seems that predominantly the anterior segment performs HCO_3^- secretion and Cl^- absorption *via* anion exchange *in vivo*. Altered chemistry of luminal content, as fluids are moving along the intestine, pose thermodynamic restraints on the anion exchange process in the distal portion of the intestine. An unavoidable consequence of the intestinal anion exchange is absorption of what appears to be an acidic fluid, which also is also likely hyperosmotic.

Future directions

(1) It appears from the relatively few studies addressing the cellular mechanisms responsible for intestinal HCO_3^- secretion that species-specific difference may exist, offering an exciting comparative field for further studies. Specifically, the likely involvement of basolateral NBC and the possible role for a basolateral H^+-pump in some species deserve attention.

(2) A close association of AE and CAII has been observed in mammalian systems and offers a possible explanation for how high cellular HCO_3^- concentrations required for apical anion exchange may arise. Studies of possible cellular and subcellular colocalization and coimmunoprecipitation demonstrating binding of CAII to AE in the marine fish intestine would provide elegant evidence for this hypothesized metabolon and its role in marine teleost osmoregulation.

(3) To date, studies of intestinal salt and water absorption in marine fish have been conducted in unfed animals. However, it seems likely that acidic stomach content containing high Cl^- concentrations (from gastric HCl secretion) may greatly influence intestinal transport as it enters the anterior intestine. In particular, anion exchange can be predicted to respond to such conditions because the acidity of the stomach content would ‘consume’ luminal HCO_3^- and increase luminal Cl^- concentrations, both providing more favorable conditions for anion exchange. Clearly studies of interactions between feeding and osmoregulation in general and the dual function of the intestine in particular are required.

(4) At least five different transport proteins or enzymes (AE, CA, NKA, NHE, NBC) are directly involved in the EA mediated transport but none of these are molecularly identified at present. Further, it is unknown which component(s) of the AE transport system are rate limiting, whether the overall activity of the system is regulated at the genomic or the posttranslational level and what environmental cues trigger such regulation. Functional genomic/proteomic approaches and immunohistological techniques seem ideally suited to address these challenging but fascinating unknowns.

(5) The acidic and hyperosmotic nature of the fluids absorbed by the marine teleost intestine is unusual and may be related to the unique and adverse composition of intestinal fluids. An investigation of the possible influence of luminal chemistry on absorbate composition, considering all electrolytes, seems timely and could involve comparisons of intestinal tissue from freshwater and seawater acclimated euryhaline fish.

(6) Whether water movement across the intestinal epithelium of marine fish occurs *via* a transcellular or paracellular route remains to be determined.

(7) Finally, although the intestinal HCO_3^- secretion is not a part of dynamic acid–base balance regulation, integrative aspects of this recently recognized intestinal transport process must be considered. It seems likely that intestinal base secretion might be regulated in response to ambient salinity and possibly altered by feeding, which inevitably have the potential to influence systemic acid–base balance and thus would require compensatory adjustments of extra-intestinal transport processes.

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