

## Review

# Carbonic anhydrase and acid–base regulation in fish

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### Summary

**Carbonic anhydrase (CA) is the zinc metalloenzyme that catalyses the reversible reactions of CO<sub>2</sub> with water. CA plays a crucial role in systemic acid–base regulation in fish by providing acid–base equivalents for exchange with the environment. Unlike air-breathing vertebrates, which frequently utilize alterations of breathing (respiratory compensation) to regulate acid–base status, acid–base balance in fish relies almost entirely upon the direct exchange of acid–base equivalents with the environment (metabolic compensation). The gill is the critical site of metabolic compensation, with the kidney playing a supporting role. At the gill, cytosolic CA catalyses the hydration of CO<sub>2</sub> to H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> for export to the water. In the kidney, cytosolic and membrane-bound CA isoforms have been implicated in HCO<sub>3</sub><sup>-</sup> reabsorption and urine acidification. In this review, the CA isoforms that have been identified to date in fish will be discussed together with their tissue localizations and roles in systemic acid–base regulation.**

Key words: carbonic anhydrase, acid–base regulation, fish, gill, kidney, gut, V-type H<sup>+</sup>-ATPase, Na<sup>+</sup>/H<sup>+</sup> exchanger, Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger, Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporter, CA isoforms.

### Introduction

Acid–base regulation in vertebrates, including fish, is inextricably linked to carbon dioxide (CO<sub>2</sub>) excretion through the reversible hydration/dehydration reactions of CO<sub>2</sub> and the acid–base equivalents H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>: CO<sub>2</sub>+H<sub>2</sub>O↔H<sup>+</sup>+HCO<sub>3</sub><sup>-</sup>. In fish, however, acid–base regulation is also coupled to ionic regulation because acid–base compensation relies primarily on the direct transfer of H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> across the gill in exchange for Na<sup>+</sup> and Cl<sup>-</sup>, respectively. Regulation of NaCl movement across the gill, in turn, is the keystone to maintaining ionic and osmotic balance in fish. A vital participant in all three processes is carbonic anhydrase (CA), the zinc metalloenzyme that catalyses the reversible hydration/dehydration reactions of CO<sub>2</sub> and that therefore is critical to CO<sub>2</sub> excretion, ionic regulation and acid–base balance. The role played by CA in acid–base balance in fish often takes a backseat to its more visible roles in CO<sub>2</sub> excretion (for reviews, see Randall and Val, 1995; Henry and Heming, 1998; Tufts and Perry, 1998; Henry and Swenson, 2000; Tufts et al., 2003; Evans et al., 2005; Esbaugh and Tufts, 2006a) and ionic regulation (for reviews, see Maetz, 1971; Maetz and Bornancin, 1975; Haswell et al., 1980; Pelis and Renfro, 2004; Evans et al., 2005; Tresguerres et al., 2006a). Thus, the objective of this paper is to highlight the contributions of CA to acid–base regulation in fish and, keeping in mind previous reviews of acid–base regulation in fish that have touched upon CA (e.g. Haswell et al., 1980; Perry and Laurent, 1990; Marshall and Grosell, 2006; Perry and Gilmour, 2006), this paper will focus primarily on recent developments in our understanding of the diversity and distribution of CA isoforms in fish as well as on emerging models of acid–base regulation.

### CA isoforms in fish

To date, 16 CA isoforms belonging to the α-CA gene family have been identified in mammals; this number may represent the full complement of mammalian α-CAs as genomic database searches

have failed to yield additional CA sequences (Hilvo et al., 2008). The mammalian α-CAs differ in molecular sequence, kinetic properties, susceptibility to inhibitors, tissue distribution and subcellular localization (Fig. 1) (for reviews, see Sly and Hu, 1995; Chegwiddden and Carter, 2000; Hewett-Emmett, 2000; Sly, 2000; Tashian et al., 2000; Schwartz, 2002; Purkerson and Schwartz, 2007; Hilvo et al., 2008). Three isoforms, VIII, X and XI, lack catalytic activity and are termed CA-related proteins (CA-RP). At least two CA-RPs have been identified in fish (Fig. 2) (Esbaugh and Tufts, 2007; Lin et al., 2008), but as in mammals, the function of CA-RPs remains unclear (Tashian et al., 2000). The mammalian enzymes with catalytic activity can be divided on the basis of subcellular localization. The intracellular CAs include five cytosolic isoforms [I, II, III, VII and XIII (Lehtonen et al., 2004) (for reviews, see Chegwiddden and Carter, 2000; Hewett-Emmett, 2000)] as well as the two CA V homologues (A and B) that, by virtue of the leader sequence they possess, are targeted to mitochondria (Fujikawa-Adachi et al., 1999b). Among the extracellular CA isoforms, three groupings can be detected. CA VI is secreted (Fernley et al., 1988), CAs IX, XII and XIV are expressed as single-pass transmembrane proteins with CA XIV normally appearing on the apical membrane, as opposed to the basolateral membrane for CA XII and CA IX (Opavský et al., 1996; Türeci et al., 1998; Mori et al., 1999; Fujikawa-Adachi et al., 1999a; Schwartz et al., 2001), and CAs IV and XV are anchored by a glycosylphosphatidylinositol (GPI) linkage to the outer leaflet of the plasma membrane and therefore typically appear on the apical membrane (Zhu and Sly, 1990; Waheed et al., 1992; Hilvo et al., 2005) (for a review, see Sly, 2000).

Homology-based cloning strategies (e.g. Peterson et al., 1997) and recent mining of genomic databases for teleost fish (e.g. Lin et al., 2008) indicate that piscine orthologues exist for many of the mammalian CA isoforms (Fig. 2). Little is known of many of these orthologues beyond their molecular sequence; much work remains

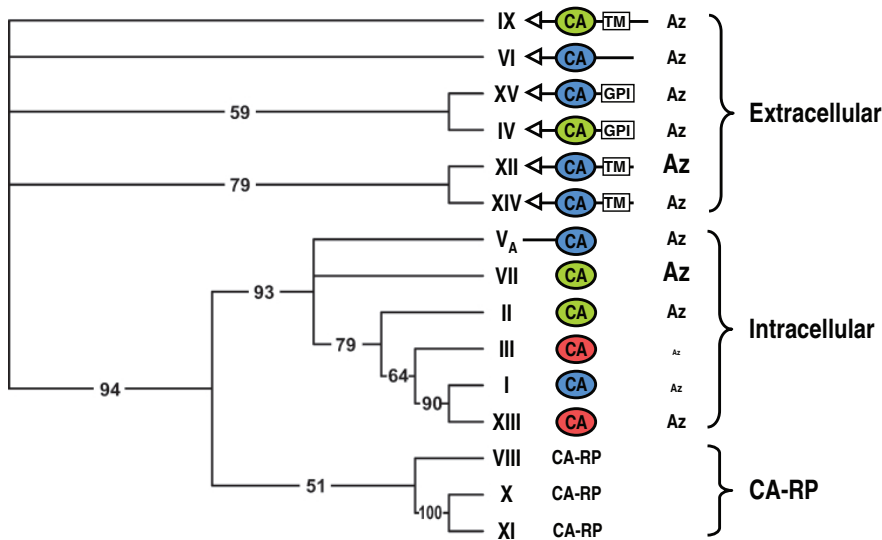


Fig. 1. A summary of the phylogenetic relationships among mammalian carbonic anhydrase (CA) isoforms illustrating their grouping into extracellular, intracellular and CA-related proteins (CA-RP). In addition, the sensitivity of the various isoforms to inhibition by the sulphonamide acetazolamide is depicted together with the domain structure of the associated genes. The phylogenetic relationships are for mouse (*Mus musculus*) CA isoforms and were taken from Hilvo et al. (Hilvo et al., 2005) (please see Hilvo et al. for details of the phylogenetic tree). Sensitivity to inhibition by acetazolamide was obtained from Hilvo et al. (Hilvo et al., 2008), and larger font indicates greater sensitivity (i.e. lower inhibition constant or  $K_i$  value). Domain structures for the various CA genes are according to Purkerson and Schwartz (Purkerson and Schwartz, 2007) with leader sequences indicated by arrows or lines, catalytic activity indicated by CA domain colour [high=green, moderate=blue and low=red (Hilvo et al., 2008)], and membrane linkage indicated as transmembrane (TM) or glycosylphosphatidylinositol (GPI).

to characterize the individual isoforms in terms of activity, inhibitor sensitivity and tissue distribution, as well as to explore interspecific diversity within a given isoform. Fish are notably different from mammals with respect to CA IV and cytosolic CA isoforms (Fig. 2). Whereas mammals possess only one CA IV isozyme, at least some teleost fish appear to express as many as nine CA IV-like isoforms. These type IV-like CAs have been grouped into three clades annotated as CA IV, CA 15 (CA IV-like group 1) and CA 16 (CA IV-like group 2), each containing a, b and c isoforms (Lin et al., 2008). At least two of the teleost CA IV-related isoforms, CA IVa and CA 15a, exhibit differences in tissue distribution in both embryonic and adult zebrafish (*Danio rerio*) (Lin et al., 2008), a finding that imbues the diversity of teleost CA IV-like isoforms with potential functional significance (see below). The question of whether fish express a CA XV-like isoform remains open. The CA IV-like isoforms identified by Lin and colleagues (Lin et al., 2008) are grouped with mammalian and teleost CA IV orthologues on the basis of sequence identity, but CA IV and XV in mammals are closely related, sharing a common domain structure. Moreover, recent work on Pacific hagfish (*Eptatretus stouti*), an agnathan fish, identified both CA IV-like and CA XV-like sequences (Esbaugh et al., 2009a). A type IV-like CA isoform has also been detected in dogfish (*Squalus acanthias*), an elasmobranch (Gilmour et al., 2007a). However, in the absence of sequence information for agnathan and elasmobranch species, whether multiple CA IV-like isoforms exist in fish groups other than teleosts remains to be determined.

Phylogenetic analyses suggest that the main cytosolic CA isoforms in fish and mammals differ. The point of divergence occurs after the appearance of CA V and CA VII, which appear in both fish and mammals (Figs 2 and 3). The I, II, III, XIII gene cluster emerged in mammals (or in the tetrapod line – our present knowledge of non-mammalian CA isoforms is insufficient to pinpoint the divergence more accurately than fish *versus* mammals). CA II is the workhorse of this cluster, being a high activity isoform of near ubiquitous distribution (Chegwidden and Carter, 2000) that contributes to systemic acid–base regulation both as the main red blood cell (RBC) isoform underlying CO<sub>2</sub> excretion (reviewed by Geers and Gros, 2000; Henry and Swenson, 2000; Swenson, 2000) and as a key player in HCO<sub>3</sub><sup>-</sup> reabsorption in the mammalian kidney, where it accounts

for 95% of renal total CA activity (reviewed by Swenson, 2000; Schwartz, 2002; Purkerson and Schwartz, 2007). By contrast, and keeping in mind that the data on which to base phylogenetic analyses are very limited, fish seem to have retained the ancestral state of a single, high activity CA isoform until the appearance of the teleosts, where a whole genome duplication of the teleost common ancestor gave rise to two closely related cytosolic isoforms differing in tissue distribution and kinetic properties (Figs 2 and 3). Our understanding of the functional significance of the two teleost isoforms rests entirely on work carried out in two species, zebrafish (Lin et al., 2008) and rainbow trout (*Oncorhynchus mykiss*) (Rahim et al., 1988; Esbaugh et al., 2004; Esbaugh et al., 2005), emphasizing a need for caution in making broad generalizations. Nevertheless, the available data indicate that mRNA for one isoform, variously termed CA II-like b or CAb, is expressed predominantly in the blood of zebrafish (Lin et al., 2008) and trout (Esbaugh et al., 2004; Esbaugh et al., 2005), whereas that of the second isoform, CA II-like a or CAa, is more widely distributed, with high expression in the gills, lower expression in the kidney and little or no RBC expression (Esbaugh et al., 2005; Lin et al., 2008). Correspondingly, immunohistochemical localization of CA in trout gills revealed the existence of two distinct isozymes, one in RBCs and the other in gill tissue (Rahim et al., 1988), with the RBC form exhibiting a higher catalytic efficiency ( $k_{cat}/K_m$ ) and greater sensitivity to the endogenous CA inhibitor present in trout plasma (Esbaugh et al., 2005). Assuming that this pattern holds across other teleost species, with the only evidence at the moment being that CAs cloned from blood have generally grouped with the ‘b’ isoforms (e.g. carp, *Cyprinus carpio*, in Fig. 3) while those cloned from gill tissue have generally grouped with the ‘a’/‘c’ isoforms (e.g. Osorezan dace, *Tribolodon hakonensis*, in Fig. 3), then teleost fish differ from mammals in making use of cytosolic CAs that have been tailored to favour CO<sub>2</sub> excretion (the higher activity RBC isoform) *versus* acid–base regulation (the slower isoform found in gill and kidney tissue). Notably, neither of these isoforms is closely related to mammalian CA II and regardless of whether the ‘a/b’ or ‘c/b’ nomenclature ultimately holds sway, fish cytosolic CA should not be termed CA II (see also Tufts et al., 2003; Esbaugh et al., 2005; Esbaugh and Tufts, 2006a; Lin et al., 2008). Evidence supporting the hypothesis that non-teleost fish express a single cytosolic isoform (beyond CA VII) is sparse. CA has been

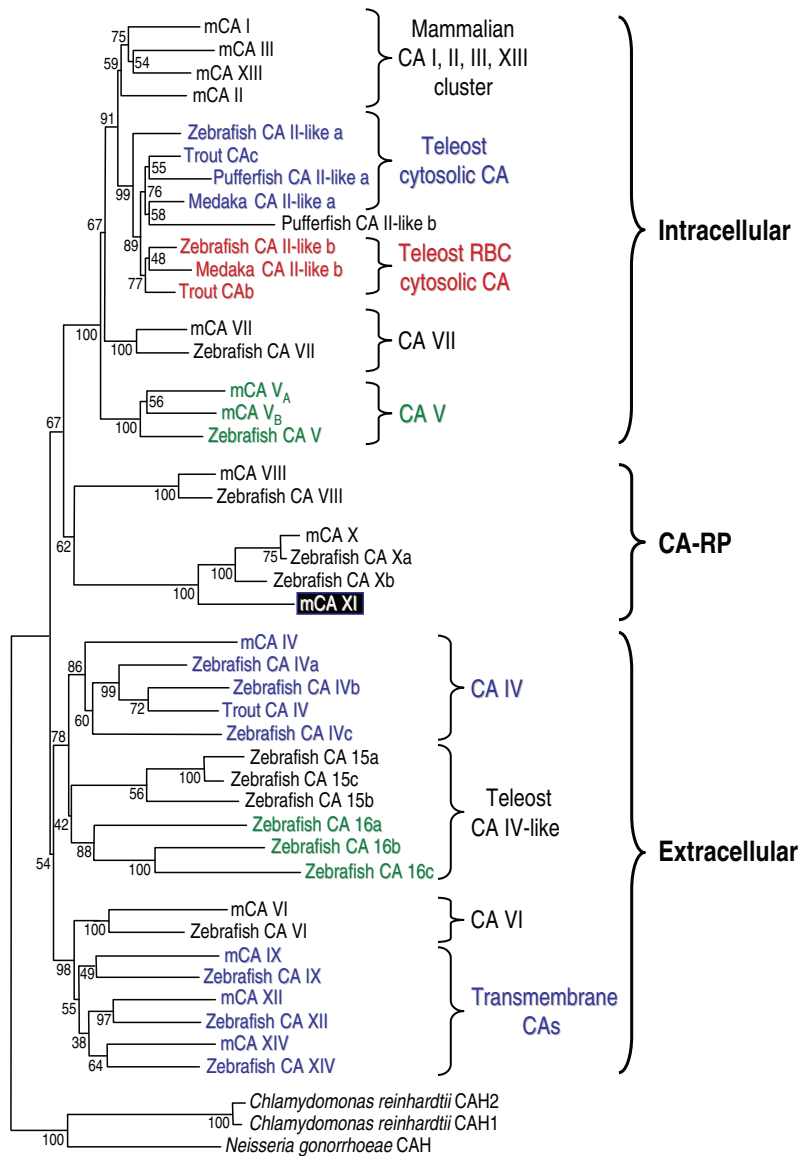


Fig. 2. A summary of the phylogenetic relationships among mammalian (mouse, *Mus musculus*; m) and fish (medaka *Oryzias latipes*; pufferfish *Tetraodon nigroviridis*; rainbow trout *Oncorhynchus mykiss*; zebrafish *Danio rerio*) CA isoforms. The original consensus tree was created by Lin et al. (Lin et al., 2008), which should be consulted for details of tree construction and bootstrap values. The original tree has been modified for this figure to emphasize the relationships between mammalian (represented by mouse) and fish isoforms and to emphasize clusters of isoforms that form groups (varying font colours have been used to highlight the members of a group). Mouse CA XI is highlighted because a fish equivalent has not been identified. CA-RP, carbonic anhydrase-related protein.

cloned from the blood of several non-teleost fish, including gar (*Lepisosteus osseus*), a holostean fish (Lund et al., 2002), dogfish, an elasmobranch (Gilmour et al., 2007a), and lamprey (*Petromyzon marinus*), an agnathan (Esbaugh and Tufts, 2006b), but only in lamprey was a systematic attempt made to determine whether additional cytosolic CA isoforms were present in other tissues (Esbaugh and Tufts, 2006b). The results of this analysis indicated that lamprey express only a single high-activity cytosolic CA isoform that groups most closely with CA VII and is found in a variety of tissues including blood and gill (Esbaugh and Tufts, 2006b). By the divergence of elasmobranchs, on the other hand, a CA isoform cloned from blood can be clearly distinguished from CA VII (Fig. 3) (Gilmour et al., 2007a). Clearly, additional sequence information together with functional characterization of fish cytosolic CAs is required to clarify these evolutionary relationships.

#### Acid–base regulation in fish: an overview

Acid–base compensation in fish relies on metabolic strategies in which acid–base equivalents are transferred between the animal and the external environment, primarily across the gill (for reviews, see

Claiborne et al., 2002; Perry et al., 2003b; Evans et al., 2005; Perry and Gilmour, 2006). This approach contrasts with that of tetrapods, where acid–base disturbances are regulated through the adjustment of ventilation (respiratory compensation) and/or urinary acid excretion (metabolic compensation). Differences in compensatory strategies between fish and mammals reflect the relatively low  $O_2$  content of the aquatic environment and resultant high convection requirement for  $O_2$  in fish (ventilation volume per unit  $O_2$  uptake). The high convection requirement for  $O_2$  constrains the capacity of fish to reduce ventilation without impacting negatively upon  $O_2$  uptake, and at the same time sets arterial  $CO_2$  tensions to low values [ $\sim 2$  Torr ( $\sim 0.27$  kPa) in fish versus  $\sim 40$  Torr (5.32 kPa) in humans], limiting the capacity of fish to raise pH by ‘blowing off’  $CO_2$  through hyperventilation (see reviews by Heisler, 1986; Swenson, 2000; Perry and Gilmour, 2006). When these constraints are relieved, as for example in the air-breathing lungfish (*Protopterus annectens*), then respiratory compensation of an acid–base disturbance may be employed (Gilmour et al., 2007b). The vast majority of fish, however, regulate pH by adjusting plasma  $HCO_3^-$  levels through the differential regulation of  $H^+$  and  $HCO_3^-$  effluxes

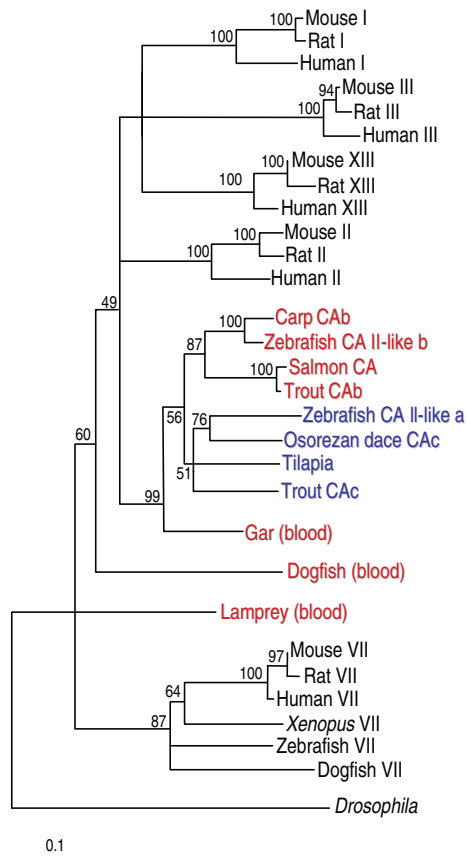


Fig. 3. A summary of the phylogenetic relationships among mammalian (human *Homo sapiens*; mouse *Mus musculus*; rat *Rattus norvegicus*) and fish (carp *Cyprinus carpio*; dogfish *Squalus acanthias*; gar *Lepisosteus osseus*; lamprey *Petromyzon marinus*; Osorezan dace *Tribolodon hakonensis*; rainbow trout *Oncorhynchus mykiss*; salmon *Salmo salar*; tilapia *Oreochromis mossambicus*; zebrafish *Danio rerio*) cytosolic CA isoforms. Redrawn from Gilmour et al. (Gilmour et al., 2007a), which should be consulted for details of tree construction and bootstrap values.

across the gill, effluxes that are coupled to the influx of, respectively,  $\text{Na}^+$  and  $\text{Cl}^-$  (Goss et al., 1992b; Claiborne et al., 2002; Perry et al., 2003a; Perry et al., 2003b; Evans et al., 2005). Compensation for a systemic acidosis is achieved by increasing branchial net acid excretion, through increased  $\text{H}^+$  excretion and/or decreased  $\text{HCO}_3^-$  excretion, so as to accumulate  $\text{HCO}_3^-$  ions. Individual species vary in their use of enhanced proton extrusion, reduced  $\text{HCO}_3^-$  loss or both. For example, whereas rainbow trout primarily reduce the rate of  $\text{Cl}^-/\text{HCO}_3^-$  exchange to regulate pH during a hypercapnic acidosis, eel (*Anguilla rostrata*) rely entirely on the stimulation of  $\text{H}^+$  loss in exchange for  $\text{Na}^+$  uptake (Hyde and Perry, 1989) and brown bullhead (*Ameiurus nebulosus*) employ both mechanisms (Goss et al., 1992a). Intriguingly, in some cases the mechanisms employed depend on whether the acidosis was of metabolic or respiratory origin. Rainbow trout, for example, respond to a hypercapnic acidosis by reducing the rate of  $\text{Cl}^-/\text{HCO}_3^-$  exchange (Hyde and Perry, 1989) but increase branchial proton extrusion when infused with HCl (Goss and Wood, 1991). Regardless of the mechanism employed,  $\text{HCO}_3^-$  accumulation would be short-circuited by urinary  $\text{HCO}_3^-$  loss in the absence of renal adjustments to enhance the reabsorption of filtered  $\text{HCO}_3^-$  (Perry and Fryer, 1997; Perry et al., 2003b; Perry and Gilmour,

2006). It is therefore not surprising that the responses of the kidney, at least in freshwater teleosts, are both significant and influenced by the type of acid–base challenge (Wood et al., 1999); the apparent insensitivity of the kidney to acid–base challenges in marine fish (Swenson, 2003) remains puzzling (see below). The kidney also plays a role in compensation for a systemic alkalosis, which involves lowering plasma  $\text{HCO}_3^-$  levels by increasing both branchial and renal base excretion (e.g. Goss and Wood, 1991; Goss and Perry, 1994; Wood et al., 1999).

Despite our basic understanding of how fish respond to acid–base challenges, the cellular and molecular mechanisms underlying the transfer of acid–base equivalents at the gill and kidney remain uncertain. There is widespread agreement that  $\text{HCO}_3^-$  and proton excretion at the gill occur in exchange for, respectively,  $\text{Cl}^-$  and  $\text{Na}^+$  uptake (Claiborne et al., 2002; Marshall, 2002; Hirose et al., 2003; Perry et al., 2003b; Evans et al., 2005), and since most fish, marine or freshwater, regulate internal NaCl levels at the gill (by excreting NaCl in a marine environment or actively taking up NaCl in fresh water), acid–base and ionic regulation in fish are tightly coupled. Marine elasmobranchs are an exception in this regard because the rectal gland is used to excrete NaCl (Shuttleworth, 1988) but acid–base disturbances are regulated at the gill (see below). CA plays a key role in both processes by providing the acid–base equivalents that function as counter-ions for NaCl movement and that are excreted to maintain or restore acid–base balance. Recent work on CA has provided insight into its importance in acid–base regulation at both the gill and kidney.

#### CA in branchial acid–base regulation

High levels of CA activity have been found in the gills of all fish species examined (reviewed by Perry and Laurent, 1990; Henry and Swenson, 2000) but as Henry and Swenson point out, the physiological role of branchial CA cannot be inferred only from its presence or activity in the gill (Henry and Swenson, 2000). CA is widely acknowledged as contributing to both ion movement and acid–base regulation, but experimental evidence in support of these functions is relatively scarce. Because the CA isoform complement of the gill seems to differ among fish groups, the role of CA in branchial acid–base regulation will be considered on a group-specific basis.

#### Teleost fish

Based on assessments of CA activity in gill homogenates, the gill has long been known as a CA-rich tissue (Maren, 1967). First using Hansson's technique, a histochemical approach employing a cobalt phosphate/cobalt sulphate vital stain (e.g. Dimberg et al., 1981; Conley and Mallatt, 1988; Flügel et al., 1991), and more recently immunohistochemical approaches with homologous antibodies (e.g. Rahim et al., 1988; Sender et al., 1999; Hirata et al., 2003; Georgalis et al., 2006b), *in situ* hybridization (Hirata et al., 2003; Georgalis et al., 2006b; Lin et al., 2008) and cell subtype isolation in conjunction with real-time RT-PCR (Nawata et al., 2007), CA has been localized to most cell types in the teleost fish gill including mitochondrion-rich (MR) cells, pavement cells, mucus cells and even neuroepithelial cells (Z. Qin, J. Lewis and S. F. Perry, unpublished observations). However, characterization of the CA isoform complement as well as the cellular and subcellular distribution of branchial CA remains a work in progress, not least because recent work has emphasized both the diversity of ionocytes present in the branchial epithelium and interspecific variation in this diversity (for reviews, see Hirose et al., 2003; Evans et al., 2005; Hwang and Lee, 2007) [as well as the review by Hwang in

this issue (Hwang, 2009)]. A further complication among euryhaline teleosts is intraspecific variation in ionocyte diversity with environmental salinity (Hirose et al., 2003; Hwang and Lee, 2007), although ionocyte heterogeneity in marine teleosts has received far less attention than is the case for freshwater species (see Hwang, 2009). Because studies of branchial CA expression in euryhaline/seawater teleost fish have tended to focus more on whether salinity changes affect CA expression or abundance (e.g. Dimberg et al., 1981; Kültz et al., 1992; Sender et al., 1999; Scott et al., 2005; Scott et al., 2008) than on the cellular distribution of cytosolic CA, our knowledge of CA isoform distribution in the gills of marine and euryhaline teleosts is very limited. Hence, the following discussion will focus on freshwater species, which have been more thoroughly investigated to date.

Among freshwater teleosts, the gills of rainbow trout (Georgalis et al., 2006b), zebrafish (Lin et al., 2008) and Osorezan dace (Hirata et al., 2003) have been found to express the teleost general cytosolic CA isoform (i.e. the 'a'/c' isoform; see above), but only in zebrafish has the absence of other intracellular isoforms, CA V and CA VII, been confirmed (Lin et al., 2008). In rainbow trout (Fig. 4A), cytosolic CA is present in both pavement and MR cells (Rahim et al., 1988; Georgalis et al., 2006b; Nawata et al., 2007) with a primarily apical localization (Rahim et al., 1988). Two populations of MR cells exist in rainbow trout and can be distinguished on the basis of whether peanut lectin agglutinin (PNA) binding sites are present on the apical membranes (Goss et al., 2001; Galvez et al., 2002). Both cell types are enriched in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (NKA), but only the  $\text{PNA}^+$  MR cells also exhibit

the extensive three-dimensional tubular network of basolateral membranes that typifies the classical freshwater 'chloride cell' (Perry, 1997).  $\text{PNA}^+$  MR cells are postulated to be responsible for base excretion (coupled to  $\text{Cl}^-$  uptake), whereas acid excretion (coupled to  $\text{Na}^+$  uptake) is proposed to occur *via*  $\text{PNA}^-$  MR cells (reviewed by Perry and Gilmour, 2006). Cytosolic CA is presumed to be present in both MR cell subtypes (Fig. 4), but this assumption remains to be confirmed experimentally. In zebrafish (Fig. 4B), at least three ionocyte subtypes probably occur (Hwang and Lee, 2007), with cytosolic CA expression having been localized to only one of the three, the proton pump-rich (HR) cell (Esaki et al., 2007; Lin et al., 2008). Zebrafish HR cells express V-type  $\text{H}^+$ -ATPase highly in the apical membrane (Lin et al., 2006) and are responsible for  $\text{Na}^+$  uptake and  $\text{H}^+$  extrusion (Lin et al., 2006; Hornig et al., 2007; Esaki et al., 2007), thereby playing a role in ion uptake and acid–base balance similar to that proposed for the rainbow trout  $\text{PNA}^-$  MR cells (Fig. 4). A second ionocyte in the zebrafish branchial epithelium, the NaR cell, is mitochondrion rich with high NKA expression localized to tubular enfoldings of the basolateral membrane (Lin et al., 2006). The NaR cell corresponds to the classical freshwater chloride cell and is analogous in many respects to the trout  $\text{PNA}^+$  MR cell, but lacks detectable cytosolic CA expression and, unlike the HR cell, does not bind the lectin concanavalin A (Esaki et al., 2007; Lin et al., 2008). Relative to NaR cells, zebrafish HR cells are poorer in mitochondria and NKA but enriched in proton pump (Lin et al., 2006; Esaki et al., 2007). The same statement holds true for trout  $\text{PNA}^-$  MR cells relative to  $\text{PNA}^+$  MR cells (Galvez et al., 2002). Despite the similarities,

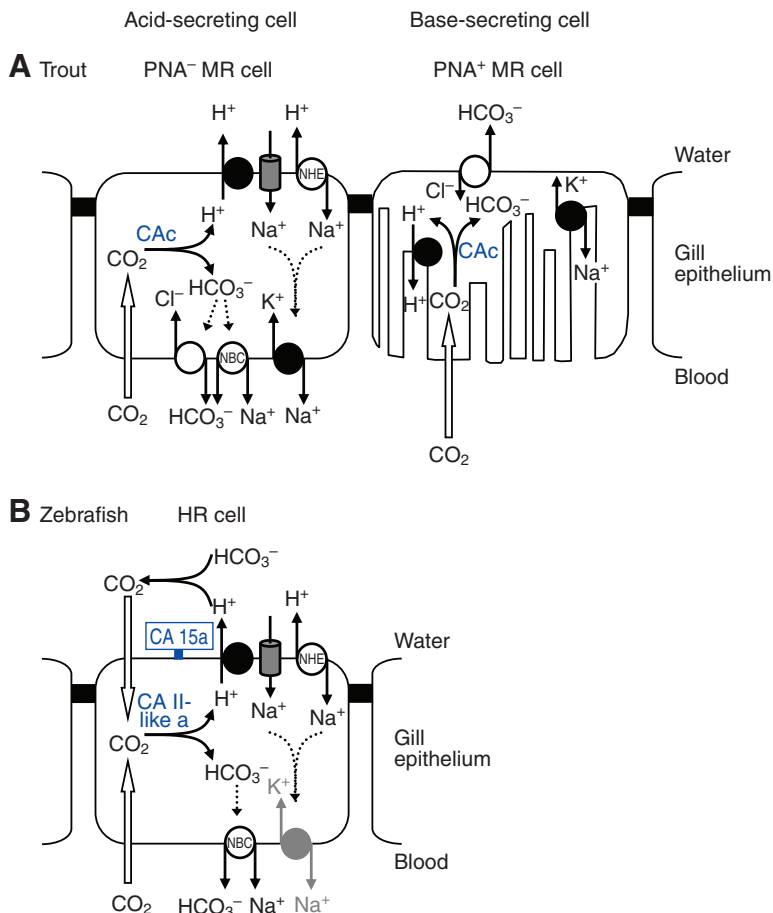


Fig. 4. Schematic representations of cell types in the (A) trout and (B) zebrafish branchial epithelium that are thought to be responsible for the excretion of acid–base equivalents (see Perry and Gilmour, 2006; Hwang and Lee, 2007) [see also Ivanis et al. (Ivanis et al., 2008b) for data that necessitate a re-evaluation of the trout model]. Electroneutral exchangers are drawn as open circles while ATPases are drawn as filled circles. The extensive basolateral tubular network of the trout  $\text{PNA}^+$  MR cell is indicated by the irregular basolateral membrane; the basolateral membranes of the trout  $\text{PNA}^-$  MR cell and zebrafish HR cell probably possess some infoldings but not to the extent observed in the trout  $\text{PNA}^+$  MR cell. The zebrafish HR cell is less enriched in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase than is another ionocyte in the branchial epithelium and this difference is indicated by the greying of the basolateral  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (see text). PNA, peanut lectin agglutinin; MR, mitochondrion rich; HR, V-type  $\text{H}^+$ -ATPase-rich; NHE,  $\text{Na}^+$ / $\text{H}^+$  exchanger; NBC,  $\text{Na}^+$ / $\text{HCO}_3^-$  cotransporter; CAC, trout general cytosolic carbonic anhydrase isoform; CA II-like a, zebrafish general cytosolic carbonic anhydrase isoform; CA 15a, zebrafish CA IV-like isoform a.

however, several key differences in transporter protein make-up distinguish trout from zebrafish ionocyte complements [discussed by Hwang in this issue (Hwang, 2009)], not least of which is the apparent difference in cytosolic CA expression. Branchial ionocyte heterogeneity has also been explored in tilapia (*Oreochromis mossambicus*) (Hwang and Lee, 2007; Hiroi et al., 2008; Hwang, 2009), but not as yet with respect to CA expression.

Most biochemical and physiological evidence suggests that branchial CA in teleost fish is restricted to the intracellular environment. Differential centrifugation of gill homogenates to isolate subcellular fractions has indicated that the vast majority of CA activity is associated with the cytoplasmic fraction (Henry et al., 1988; Henry et al., 1993; Henry et al., 1997; Gervais and Tufts, 1998; Sender et al., 1999; Gilmour et al., 2001; Gilmour et al., 2002; Tufts et al., 2002). The low level of CA activity detected in membrane fractions has usually (e.g. Gervais and Tufts, 1998; Gilmour et al., 2001; Gilmour et al., 2002), but not always (e.g. Tufts et al., 2002), failed to pass tests considered diagnostic of membrane-associated CA, such as resistance to SDS (Waheed et al., 1996) or release of CA IV from its membrane anchorage by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) (Zhu and Sly, 1990). Physiological experiments making use of a stopflow approach have also supported the idea that branchial CA in teleost fish is exclusively intracellular. In the stopflow approach, a change in pH upon halting the flow of a solution is taken to be indicative of CO<sub>2</sub> reactions that are out of equilibrium because of insufficient access to CA activity (Gilmour, 1998b). Blood leaving the gills of rainbow trout exhibits a disequilibrium pH that can be eliminated by the addition of exogenous CA to the plasma, indicating that plasma CO<sub>2</sub> reactions do not have access to CA activity during passage through the gills (Gilmour et al., 1994) (reviewed by Gilmour, 1998a) (see also Randall, 1982). Similarly, use of the stopflow approach suggested that CO<sub>2</sub> reactions in water passing across the gills of rainbow trout do not have access to CA activity (Heming, 1986; Perry et al., 1999) [the conflicting results of a separate stopflow study on rainbow trout (Wright et al., 1986) have been attributed to methodological considerations (Henry and Heming, 1998)]. Given this context, it is interesting that Lin and colleagues (Lin et al., 2008) recently localized mRNA expression for the CA IV-like isoform, CA 15a, to the HR cells of the zebrafish gill. This finding is not, however, necessarily inconsistent with the data of other studies. In particular, it is unclear whether the biochemical and physiological approaches used in previous studies would be sufficiently sensitive to detect CA activity that is associated with a specific and relatively uncommon cell type within the branchial epithelium, as is postulated for zebrafish HR cell CA 15a. The recent work of Lin and colleagues (Lin et al., 2008) on zebrafish branchial CA emphasizes a pressing need to re-evaluate the rainbow trout model in greater detail with respect to the cellular distribution of CA isoforms.

Does branchial CA contribute to acid–base regulation in teleost fish? Surprisingly few studies have attempted to address this question directly. More have instead focused on the question of whether branchial CA contributes to ionic regulation, finding support for such a role by using acetazolamide or other permeant CA inhibitors to demonstrate a significant reduction in Na<sup>+</sup> [goldfish, *Carassius auratus* (Maetz, 1956; Maetz and Garcia-Romeu, 1964); rainbow trout (Kerstetter et al., 1970; Payan et al., 1975); zebrafish (Boisen et al., 2003; Esaki et al., 2007)] or Cl<sup>-</sup> uptake [goldfish (Maetz and Garcia-Romeu, 1964); tilapia (Chang and Hwang, 2004); zebrafish (Boisen et al., 2003)] when CA is inhibited (for reviews, see Perry and Laurent, 1990; Evans et al.,

2005; Tresguerres et al., 2006a). Somewhat unexpectedly, elimination of cytosolic CA or CA 15a activity from HR cells using the antisense oligonucleotide morpholino approach (to selectively knockdown translation of the CA isoform of interest) increased rather than decreased Na<sup>+</sup> influx in zebrafish larvae (Lin et al., 2008). These apparently anomalous results probably reflect compensatory mechanisms initiated in the developing embryo in the absence of cytosolic CA or CA 15a activity; for example, morphants exhibited increased expression of the Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 (Lin et al., 2008). Additional support for the role of branchial CA in ion uptake has come from the observation that CA mRNA expression [cytosolic CA (Craig et al., 2007); CA 15a (Lin et al., 2008)] as well as cytosolic CA protein levels (Perry and Laurent, 1990; Chang and Hwang, 2004) increased in soft, low Na<sup>+</sup> or low Cl<sup>-</sup> water, conditions under which ion uptake mechanisms must be enhanced if ion balance is to be maintained.

Given that cytosolic CA contributes to ion uptake by catalysing CO<sub>2</sub> hydration to provide H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> as counter-ions for Na<sup>+</sup> and Cl<sup>-</sup> uptake, respectively, it is reasonable to assume that CA will also contribute to acid–base regulation, although the experimental data to support this assumption are somewhat meager. Using an *in vivo* approach in rainbow trout, Georgalis and colleagues (Georgalis et al., 2006b) demonstrated a significant reduction in branchial net acid excretion following treatment with the permeant CA inhibitor acetazolamide, a finding that clearly implicates branchial CA in acid–base regulation. Moreover, in keeping with the role of enhanced branchial net acid excretion during compensation for a systemic acidosis (see above), the effect of acetazolamide treatment on branchial net acid excretion was stronger in trout exposed to environmental hypercapnia (Georgalis et al., 2006b). CA has also been implicated in net acid excretion by zebrafish embryos and larvae. Using a non-invasive H<sup>+</sup>-selective microelectrode to probe H<sup>+</sup> activity at the surface of zebrafish embryos/larvae, Lin and colleagues (Lin et al., 2008) were able to measure pH gradients indicative of proton extrusion by the embryo or larva. Proton extrusion was significantly reduced in morphants in which cytosolic CA translation was knocked down, as expected based on the proposed role of cytosolic CA in providing H<sup>+</sup> for export from the cell (Fig. 4B). Proton extrusion was significantly enhanced at 24 h in morphants in which CA 15a translation was knocked down, but impaired by 96 h. Increased proton extrusion is in agreement with the model in which apically localized CA 15a catalyses the conversion of extruded H<sup>+</sup> to CO<sub>2</sub>, thereby reducing external H<sup>+</sup> activity (Fig. 4B); the subsequent reversal of this effect by 96 h was attributed to compensatory changes in the expression of other H<sup>+</sup> and/or Na<sup>+</sup> transport mechanisms (Lin et al., 2008). Assessment of CA mRNA expression and/or protein during acid–base challenges has provided some additional support for the role of branchial CA in acid–base regulation in teleost fish. For example, branchial cytosolic CA mRNA expression was increased in rainbow trout exposed to hypercapnia (Georgalis et al., 2006b) and Osorezan dace exposed to acidic water (Hirata et al., 2003), while branchial CA 15a mRNA expression was increased in zebrafish exposed to acidic pH (Lin et al., 2008). Georgalis and colleagues (Georgalis et al., 2006b) also detected an increase in branchial cytosolic CA protein levels, but, interestingly, not branchial CA activity, in hypercapnic rainbow trout. Interpretation of the data for rainbow trout is complicated by the likelihood that CA is involved in both acid excretion by PNA<sup>-</sup> MR cells and base excretion by PNA<sup>+</sup> MR cells (Fig. 4); both cell subtypes will be present in gill homogenates but are expected to respond to acid–base challenges in opposite fashions. Taken as a whole, the

data indicate that acid–base fluxes are affected by loss of branchial CA activity and that regulation of branchial CA expression is sensitive to acid–base challenges, findings that strongly endorse the role of branchial CA in acid–base regulation in freshwater teleost fish.

Electroneutral exchanges of  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  for the purposes of acid–base regulation are also postulated to occur at the marine teleost fish gill, despite the fact that such exchanges are likely to augment the  $\text{NaCl}$  burden faced by these hypo-osmotic regulators (Claiborne et al., 2002; Evans et al., 2005). A role for CA in providing  $\text{H}^+$  and  $\text{HCO}_3^-$  ions for such exchanges is expected, although no significant impact of acetazolamide treatment on net acid excretion was detected in mudskipper (*Periophthalmodon schlosseri*) (Wilson et al., 2000a). The cellular location(s) of the exchange mechanisms and associated CA as well as the contribution of CA to acid–base regulation require investigation.

#### Elasmobranch fish

The gills of elasmobranch fish appear to express at least two CA isoforms. Differential centrifugation of gill homogenates revealed that the CA activity associated with membrane fractions in elasmobranch gills was substantially higher than that in teleost gills (Henry et al., 1997; Gilmour et al., 2001; Gilmour et al., 2002), was resistant to inhibition by SDS (Gilmour et al., 2002), and could be released from its membrane anchorage by treatment with PI-PLC (Gilmour et al., 2001; Gilmour et al., 2002), suggesting the presence of a type IV-like CA isoform. Subsequently, CA IV was cloned from dogfish gill tissue and localized largely to pillar cells by *in situ* hybridization (Gilmour et al., 2007a). Immunohistochemistry using an antibody raised against trout CA IV established a plasma membrane localization for dogfish CA IV (Gilmour et al., 2007a). Anchored to the plasma membrane of pillar cells, CA IV would be available to catalyse plasma  $\text{CO}_2$  reactions (Wilson et al., 2000b), specifically to dehydrate plasma  $\text{HCO}_3^-$ , in keeping with physiological data supporting a role for branchial extracellular CA in contributing to  $\text{CO}_2$  excretion in dogfish (Gilmour et al., 2001; Gilmour and Perry, 2004). In the same fashion, branchial CA IV could factor in acid–base regulation, particularly in the clearance of a  $\text{HCO}_3^-$  load (Fig. 5). Preliminary

evidence suggests that this is, indeed, the case. Clearance of an infused  $\text{NaHCO}_3$  load was slowed significantly by treatment of dogfish with polyoxyethylene-aminobenzolamide (F3500) (Swenson et al., 1995; Swenson et al., 1996), a CA inhibitor that is restricted by virtue of its molecular size to the extracellular compartment and therefore selectively inhibits extracellular CA activity (Conroy et al., 1996). Branchial extracellular CA appears to be restricted to the vascular compartment, as stopflow experiments indicated that  $\text{CO}_2$  reactions in water passing across the gills of dogfish do not have access to CA activity (Perry et al., 1999).

The majority of branchial CA activity in elasmobranchs, however, is cytoplasmic (Henry et al., 1997; Gilmour et al., 2002). Studies using heterologous antibodies suggest that cytoplasmic CA is found in most cell types of the branchial epithelium (Wilson et al., 2000b; Tresguerres et al., 2007b), including the distinct populations of NKA-rich and V-type  $\text{H}^+$ -ATPase-rich cells that have been described in the branchial epithelia of several elasmobranch species over the last few years (Piermarini and Evans, 2001; Tresguerres et al., 2005). Current models of acid–base regulation for elasmobranch fish (Fig. 5) (Piermarini and Evans, 2001) (for reviews, see Claiborne et al., 2002; Evans et al., 2004; Evans et al., 2005) postulate that these cell subtypes are responsible for acid excretion and base excretion, respectively (note that salt secretion in marine elasmobranchs relies on the rectal gland). Acid excretion is accomplished through an apical NHE (Edwards et al., 2002; Choe et al., 2005) driven by the inwardly directed  $\text{Na}^+$  gradient, with  $\text{Na}^+$  removal from the cell *via* the basolateral NKA. Base excretion reflects the coordinated actions of an apical anion exchanger, possibly a pendrin-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (Piermarini et al., 2002; Evans et al., 2004), and V-type  $\text{H}^+$ -ATPase that normally resides in cytoplasmic vesicles but is trafficked to the basolateral membrane during blood alkalosis (Tresguerres et al., 2005; Tresguerres et al., 2006c). As in models of acid–base regulation for the teleost fish gill, cytosolic CA is proposed to contribute to acid or base excretion by catalysing the hydration of  $\text{CO}_2$  to  $\text{H}^+$  and  $\text{HCO}_3^-$ . Measurements of acid–base or ion fluxes following CA inhibition under control conditions or during an acid–base challenge have been used to test this hypothesis. In acetazolamide-treated *Scyliorhinus canicula*,  $\text{Na}^+$  influx was

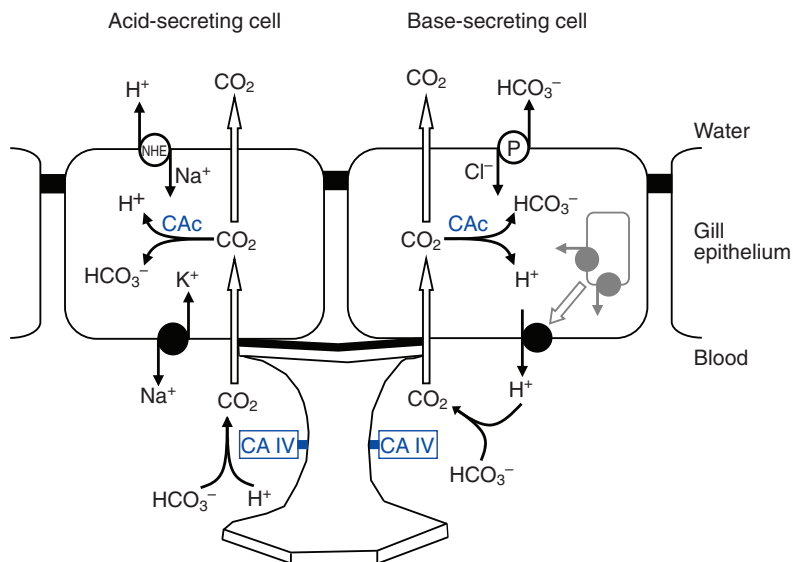


Fig. 5. A schematic representation of the cell types in the dogfish branchial epithelium that are thought to be responsible for the excretion of acid–base equivalents (see Piermarini and Evans, 2001; Tresguerres et al., 2006c), as well as the underlying pillar cells that express CA IV (see Gilmour et al., 2007a). Electroneutral exchangers are drawn as open circles while ATPases are drawn as filled circles. The basolateral membranes of the acid- and base-secreting cells are depicted as smooth but probably possess moderate infoldings (Evans et al., 2005). In the base-secreting cell, V-type  $\text{H}^+$ -ATPase is trafficked to the basolateral membrane from cytoplasmic vesicles in response to systemic alkalosis (Tresguerres et al., 2006c); this process is indicated by the greyed vesicle and arrow. NHE,  $\text{Na}^+/\text{H}^+$  exchanger; P, pendrin; CAc, cytosolic carbonic anhydrase; CA IV, dogfish carbonic anhydrase IV.

significantly reduced, supporting a role for branchial CA in acid excretion coupled to  $\text{Na}^+$  uptake (Payan and Maetz, 1973). Using changes in plasma  $\text{HCO}_3^-$  concentrations during or following infusion of a  $\text{NaHCO}_3$  load as an index of  $\text{HCO}_3^-$  excretion, inhibition of branchial CA was found to attenuate recovery from a metabolic alkalosis (Hodler et al., 1955; Swenson and Maren, 1987; Tresguerres et al., 2007b). Moreover, the  $\text{HCO}_3^-$  load was cleared more slowly during inhibition of both intracellular and extracellular branchial CA than when extracellular CA alone was selectively inhibited, an observation that intimates distinct yet additive functions for cytosolic and extracellular CA in base excretion (Swenson et al., 1995; Swenson et al., 1996). Extracellular CA could, for example, facilitate entry of  $\text{HCO}_3^-$  into gill epithelial cells by catalysing its conversion to the more diffusible  $\text{CO}_2$ . Recently, an additional role was proposed for CA in acid–base regulation in elasmobranchs. Base-infused dogfish treated with acetazolamide failed to exhibit basolateral membrane localization of  $\text{H}^+$ -ATPase, implicating CA in the mechanism that mediates  $\text{H}^+$ -ATPase translocation during a systemic alkalosis (Tresguerres et al., 2007b).

#### Agnathan fish

The lampreys include a number of anadromous species in which both marine and freshwater life stages occur. The ionoregulatory and osmoregulatory problems faced by these species are similar to those encountered by marine or freshwater teleost fish, and branchial mechanisms analogous to those of teleost fish are thought to be involved in maintaining ionic and osmotic balance, even though the cellular composition of the branchial epithelium in lamprey differs from that of teleosts (Karnaky, 1998; Bartels and Potter, 2004; Evans et al., 2005). Branchial CA in lamprey is thought to be exclusively cytoplasmic; there is no evidence to date for membrane-associated or extracellular branchial CA (Henry et al., 1993; Tufts and Perry, 1998). Unlike the situation in teleost fish, where distinct blood and gill cytosolic CA isoforms appear to exist (see above), only one cytosolic CA has been detected in lamprey (Esbaugh and Tufts, 2006b). Recent work indicates that the lamprey cytosolic CA is a high activity isoform that is unusually resistant to inhibition by acetazolamide (Esbaugh and Tufts, 2006b). Using heterologous antibodies, CA has been localized in the gills of two lamprey species (*Geotria australis* and *P. marinus*) to a population of  $\text{H}^+$ -ATPase-enriched MR cells (Choe et al., 2004; Reis-Santos et al., 2008) where it is presumed to catalyse the hydration of  $\text{CO}_2$  to supply the proton pump with  $\text{H}^+$  in a model of acid–base regulation similar to that of teleost or elasmobranch fish (Choe et al., 2004). Experimental data in support of this model, including the involvement of CA, are needed, however.

The gills of hagfish, which are stenohaline marine osmoconformers, contain MR cells that have been proposed to function in acid–base regulation as there is little requirement for ionic or osmotic regulation (Mallatt et al., 1987). Acid–base regulation in hagfish, as in other fish, appears to involve branchial  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchanges (Evans, 1984), and several of the transporters that have been implicated in driving these exchanges in teleost and elasmobranch fish, including NHE, V-type  $\text{H}^+$ -ATPase and NKA, have been detected in the hagfish gill using heterologous antibodies (Tresguerres et al., 2006b), real-time PCR (Edwards et al., 2001) or histochemical approaches (Mallatt et al., 1987). Interestingly and unlike the models that have been developed for teleosts, elasmobranchs and lamprey, all three transporters appear to be expressed in a single cell type that is presumed to be the MR cell (Tresguerres et al., 2006b). The

generation of appropriate responses to acidosis *versus* alkalosis would, under these circumstances, probably involve trafficking of transporters between cytoplasmic vesicles and the apical or basolateral membrane (Tresguerres et al., 2006b), a possibility that is supported by changes in the membrane abundance of these transporters in acid- or base-infused hagfish (Parks et al., 2007; Tresguerres et al., 2007a). In the emerging hagfish model, cytosolic CA would play its usual role of catalysing  $\text{CO}_2$  hydration to provide the acid–base equivalents for exchange. Biochemical analysis of hagfish gill homogenates revealed that most CA activity was cytoplasmic (Esbaugh et al., 2009a), and CA has been localized to hagfish branchial MR cells by histochemistry (Mallatt et al., 1987), although no isoform information is yet available for hagfish cytosolic CA. Recently, two extracellular CA isoforms were cloned from hagfish tissues and localized to gill tissue by real-time PCR (Esbaugh et al., 2009a). The mRNA expression of a CA XV-like isoform was widespread and included the gill. By contrast, mRNA for a CA IV-like isoform was expressed only in the gill, where its expression was 6-fold higher than that of the CA XV-like isoform (Esbaugh et al., 2009a). Although the cellular location and physiological function of these isoforms remain to be determined, it is tempting to speculate that one or both may contribute to  $\text{CO}_2$  excretion and acid–base regulation in fashions similar to that of pillar cell CA IV in the elasmobranch gill (see above). In this regard, it is notable that CA was localized to hagfish gill pillar cells using Hansson's (histochemical) technique (Mallatt et al., 1987).

#### CA in renal acid–base regulation

##### Localization of CA in the kidney

There are inherent difficulties associated with specifically measuring renal CA activities and mRNA/protein expression that have led to considerable confusion as to the presence or absence of CA in the fish kidney. Because the fish kidney contains haematopoietic cells and an extensive blood supply, it has been challenging to distinguish CA activity that is strictly associated with the renal nephrons from other tissues enriched with CA. Although urine acid–base status in freshwater fish is influenced by *in vivo* administration of CA inhibitors (Hodler et al., 1955; Maren, 1967; Nishimura, 1977; Georgalis et al., 2006a), similar treatment has no effect in marine fish (Hodler et al., 1955; Maren et al., 1992; Swenson, 2003). Indeed, until the finding of CA-mediated  $\text{SO}_4^{2-}$  excretion (Renfro et al., 1999), it was believed that the kidney of marine fish lacked CA activity entirely. Although relatively few species have been examined, the available data derived from studies using isolated tubules or employing immunocytochemistry suggest that CA is present in the kidneys of both marine and freshwater fish (Renfro et al., 1999; Pelis et al., 2003; Pelis and Renfro, 2004; Georgalis et al., 2006a; Gilmour et al., 2007a).

The predominant CA isoform in trout kidney is the general cytosolic variant (tCAc) (Esbaugh et al., 2005). Although previous studies have reported the presence of 'CA II' in fish kidney [e.g. Osorezan dace (Hirata et al., 2003); winter flounder (*Pleuronectes americanus*) (Pelis et al., 2003)], this renal CA is probably more appropriately termed cytosolic CA ('c' or 'a' nomenclature; see above). In trout and flounder, cytosolic CA is found within proximal tubules where it immunolocalizes to the cytosol and brush border membranes (Pelis and Renfro, 2004; Georgalis et al., 2006a) (Fig. 6). In trout kidney, the distal tubule appears to be a more important location of renal CAc expression (Georgalis et al., 2006a). On the basis of the different migration patterns of purified cytosolic CA and crude protein on non-denaturing gels, Pelis and colleagues (Pelis et al., 2003) have suggested that a sub-fraction of



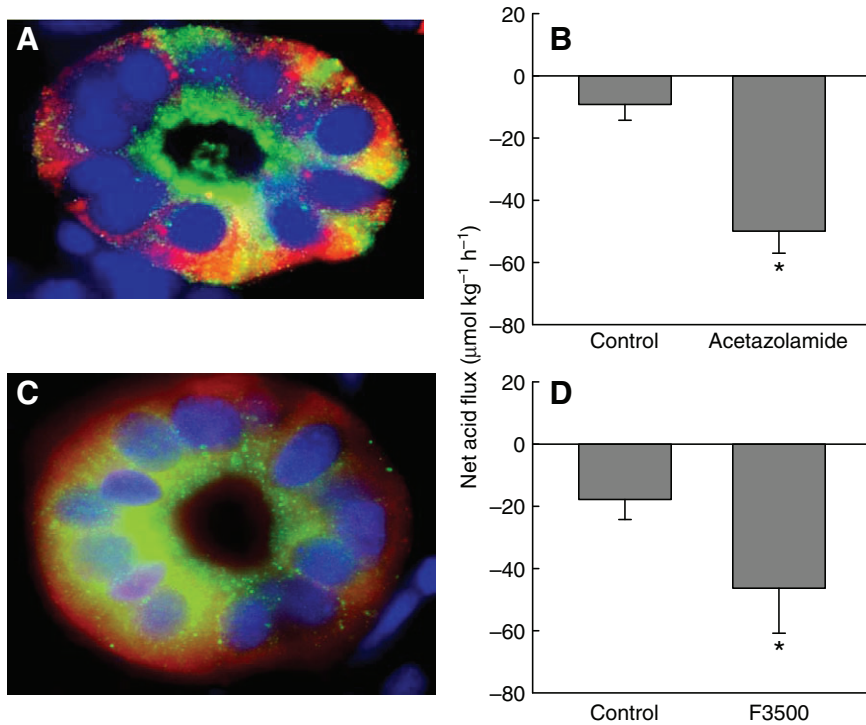


Fig. 6. Immunolocalization of (A) cytosolic carbonic anhydrase (CAc) and (C) CA IV in renal proximal tubules of rainbow trout (*Oncorhynchus mykiss*); in each micrograph CA is depicted by green fluorescence, Na<sup>+</sup>,K<sup>+</sup>-ATPase by red fluorescence and nuclei by blue fluorescence. Inhibition of (B) total CA activity with acetazolamide or (D) only extracellular CA activity with F3500 significantly reduced net urinary acid excretion (negative values indicate net base loss; asterisks indicate statistically significant differences between control and treatment values). Data were obtained from Georgalis et al. (Georgalis et al., 2006a).

CA in flounder proximal tubules is directly associated with membranes. Additionally, two membrane-specific CA isoforms have been identified in the fish kidney, CA IV in rainbow trout (Georgalis et al., 2006a) and CA IX in zebrafish (Esbaugh et al., 2009b). In trout, CA IV is localized specifically to the apical and basolateral membranes of proximal tubule cells (Georgalis et al., 2006a) (Fig. 6).

#### Role of the kidney in acid–base regulation

Although a fair bit of interspecific variability exists, the freshwater fish kidney generally plays a significant role in regulating systemic acid–base disorders (Wood and Caldwell, 1978; Kobayashi and Wood, 1980; Cameron, 1980; McDonald and Wood, 1981; Cameron and Kormanik, 1982; Wheatly et al., 1984; Perry et al., 1987; Wood, 1988; Ferguson and Boutilier, 1989; Curtis and Wood, 1992; Maren et al., 1992). A few studies (Wood and Caldwell, 1978; McDonald and Wood, 1981) have reported that changes in renal net H<sup>+</sup> excretion could account for 100% of acid–base compensation, but it is generally accepted that the kidney contributes about 5–30% to altered rates of whole body H<sup>+</sup> excretion during systemic pH changes. Thus, the major route of regulated H<sup>+</sup> secretion during acid–base disorders is *via* the gill (see above). The majority of studies on renal acid–base regulation have been conducted on freshwater species that produce large volumes of dilute urine. Marine fish (teleosts and elasmobranchs) produce small quantities of urine containing elevated levels of Ca<sup>2+</sup> and Mg<sup>2+</sup> salts. The low urine volumes coupled with an apparent requirement to acidify the urine to prevent the formation of Ca<sup>2+</sup> and Mg<sup>2+</sup> precipitates are likely key factors constraining the participation of the marine kidney in acid–base balance (Hickman and Trump, 1969). McDonald and colleagues (McDonald et al., 1982) reported an insignificant contribution of the kidney of marine lemon sole (*Parophrys vetulus*) to regulating metabolic acidosis. The kidney of marine sculpin (*Myoxocephalus octodecimspinosus*), while contributing significantly to the regulation of metabolic

acidosis, is not involved in regulating metabolic alkalosis following infused loads of NaHCO<sub>3</sub> (Maren et al., 1992). Interestingly, the imposition of a NaHCO<sub>3</sub> load in sculpin was associated with a marked alkalization of the urine without any detected increase in urinary precipitates (Maren et al., 1992). Thus, the requirement to produce acidic urine to prevent the formation of precipitates may not be as strict as originally thought. Unlike in teleosts, the kidney of elasmobranchs appears to produce urine of fixed acidity regardless of the prevailing blood acid–base status (Hodler et al., 1955; Cross et al., 1969; Swenson and Maren, 1986).

As noted above, fish generally regulate blood pH exclusively through metabolic processes. For example, during respiratory acidosis, blood pH is regulated at constant  $P_{\text{CO}_2}$  by a gradual accumulation of plasma HCO<sub>3</sub><sup>-</sup> (Heisler, 1984) achieved largely through altered rates of branchial ion exchange. Such a strategy can be effective only if the filtered HCO<sub>3</sub><sup>-</sup> is reabsorbed, a process requiring increased renal H<sup>+</sup> secretion. Thus, the successful metabolic compensation of respiratory acidosis can only be achieved if renal H<sup>+</sup> secretion is increased to match the increasing filtered HCO<sub>3</sub><sup>-</sup> load (Wood and Jackson, 1980). Depending on the species and the nature of the respiratory disturbance, plasma HCO<sub>3</sub><sup>-</sup> levels may exceed 70 mmol l<sup>-1</sup> in partially compensated animals (McKenzie et al., 2003), resulting in a massive increase in filtered HCO<sub>3</sub><sup>-</sup> load that necessitates an equally massive increase in H<sup>+</sup> secretion.

#### Renal H<sup>+</sup> secretion and HCO<sub>3</sub><sup>-</sup> reabsorption

The predominant site of renal H<sup>+</sup> secretion and HCO<sub>3</sub><sup>-</sup> reabsorption in teleost fish, as in the mammalian nephron, is thought to be the proximal tubule; the putative mechanisms are depicted in Fig. 7. The addition of H<sup>+</sup> to the filtrate is achieved by at least two mechanisms, electroneutral exchange with Na<sup>+</sup> or active H<sup>+</sup> pumping (Perry et al., 2003a; Perry et al., 2003b; Perry and Gilmour, 2006). Available evidence suggests that electroneutral Na<sup>+</sup>/H<sup>+</sup> exchange is accomplished by NHE3 (SLC9A3), a member

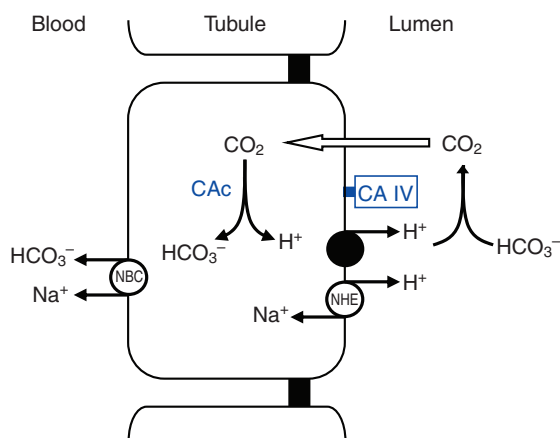


Fig. 7. A schematic representation of  $\text{HCO}_3^-$  reabsorption by the teleost kidney proximal tubule. Luminal CA isoform IV (and/or another membrane-associated isoform) catalyses the combination of filtered  $\text{HCO}_3^-$  with  $\text{H}^+$  provided by  $\text{Na}^+/\text{H}^+$  exchange (NHE) or  $\text{H}^+$ -ATPase (ATPases are drawn as filled circles). The  $\text{CO}_2$  formed by the dehydration reaction diffuses into the cytosol where cytoplasmic CA (CAc) catalyses its hydration to  $\text{HCO}_3^-$  and  $\text{H}^+$ . The  $\text{HCO}_3^-$  exits across the serosal membrane via the  $\text{Na}^+/\text{HCO}_3^-$  cotransporter isoform 1 (NBC1) and the  $\text{H}^+$  refuels the NHE or  $\text{H}^+$ -ATPase.

of the NHE (SLC9) multi-gene family (Hirata et al., 2003; Ivanis et al., 2008a) while active  $\text{H}^+$  secretion is driven by the V-type  $\text{H}^+$ -ATPase (Perry and Fryer, 1997; Perry et al., 2000; Perry et al., 2003a; Hirata et al., 2003). The mRNA levels for both the V-ATPase and NHE3 genes are increased during acidification induced by hypercapnia (Perry et al., 2003a; Ivanis et al., 2008a) or exposure to water of low pH (Hirata et al., 2003). The  $\text{H}^+$  ions fuelling the V-ATPase and NHE are derived from the hydration of intracellular  $\text{CO}_2$  in the presence of cytosolic CA. Once in the filtrate, the  $\text{H}^+$  combines with filtered  $\text{HCO}_3^-$  to form  $\text{CO}_2$ , a reaction catalysed by membrane-associated CA IV (Georgalis et al., 2006a) which is oriented to face the extracellular compartment (Fig. 7). The  $\text{CO}_2$  then diffuses into the tubule and is hydrated in the presence of CA to re-fuel the apical membrane  $\text{H}^+$  secretory mechanisms and to provide  $\text{HCO}_3^-$  to be 'reabsorbed' across the basolateral membranes via the  $\text{Na}^+/\text{HCO}_3^-$  co-transporter isoform 1 (NBC1) (Hirata et al., 2003; Perry et al., 2003a). In trout, the levels of renal NBC1 mRNA are increased during hypercapnic acidosis, presumably to match the increased rates of  $\text{HCO}_3^-$  reabsorption (Perry et al., 2003a).

The most direct evidence for an important role of renal CA in  $\text{H}^+$  secretion and  $\text{HCO}_3^-$  reabsorption comes from *in vivo* studies employing selective CA inhibitors under resting conditions and periods of acid-base disturbance. Thus, the inhibition of total CA activity in rainbow trout using acetazolamide caused a significant decrease in renal net acid excretion (see Fig. 6) that was associated with a rise in urine pH (Georgalis et al., 2006a). In that same study, the inhibition of total renal CA activity during compensated hypercapnic acidosis was associated with marked increases in urinary losses of  $\text{Na}^+$  and  $\text{HCO}_3^-$  (Georgalis et al., 2006a). The fact that levels of  $\text{HCO}_3^-$  in the urine increased from approximately 7 to  $30\text{mmol l}^{-1}$  after CA inhibition in hypercapnic fish clearly reveals the critical roles of the kidney (and renal CA) in preventing the loss of  $\text{HCO}_3^-$  that has accumulated largely by adjustment of branchial ion transfers (see above). The use of a membrane-permeant inhibitor such as acetazolamide does not permit an

assessment of the relative roles of the cytosolic and extracellular isoforms of renal CA. Georgalis and colleagues (Georgalis et al., 2006a), however, used F3500 (Conroy et al., 1996) to selectively inhibit renal extracellular CA in trout. Although too large to cross cell membranes, the relatively low molecular mass (3500Da) of F3500 allows it to be filtered, thereby providing it with access to luminal extracellular CA. Using this approach, it was demonstrated that renal luminal extracellular CA was playing a significant role in the reabsorption of filtered  $\text{HCO}_3^-$  in hypercapnic trout although to a lesser extent than the cytosolic isoform (Georgalis et al., 2006a). The proposed role of luminal extracellular CA in the dehydration of filtrate  $\text{HCO}_3^-$  is depicted in Fig. 7. Based on immunocytochemical data, the CA IV isoform is implicated in catalysing the dehydration of filtrate  $\text{HCO}_3^-$  but it is uncertain whether other membrane-associated isoforms are also involved (e.g. CA XII or XIV).

The relatively few studies that have examined the effects of acid-base disturbances on renal CA expression have yielded conflicting or ambiguous data. In rainbow trout, respiratory acidosis was accompanied by increasing mRNA and protein expression of CAc; CA IV was unaffected (Georgalis et al., 2006a). Similarly, Hirata and colleagues (Hirata et al., 2003) demonstrated increased renal expression of cytosolic CA in Osorezan dace exposed to acidic water. Lungfish, while exhibiting a significant renal component to the compensation of metabolic acidosis or alkalosis, showed no changes in renal CA mRNA expression (Gilmour et al., 2007b). The absence of any change in renal mRNA levels for cytosolic CA during metabolic acidosis is surprising given that similar treatment in mammals causes a marked increase in the expression of CA II (Tsuruoka et al., 1998). Several explanations can be offered for the lack of an effect in the lungfish: the sampling time may have been inappropriate thus preventing detection of a transient change in mRNA expression; or CA was being modified post-translationally. Alternatively, it is conceivable that CA activity was already sufficient to cope with the imposed acid-base disturbances. Because it is relatively easy to measure CA activity in tissues (Henry, 1981) concomitantly with estimating protein (western blotting) and mRNA levels (real-time PCR), researchers are encouraged to measure all three parameters to allow a complete assessment of the underlying mechanisms contributing to changes in CA activity. For tissues such as kidney, however, such an integrated approach is not always feasible because of the difficulty in eliminating the contribution of haematopoietic cells or RBCs to measurements of CA activity or protein (unless isoform-specific antibodies are used).

The important role of CA in promoting renal  $\text{H}^+$  secretion and  $\text{HCO}_3^-$  reabsorption in freshwater teleosts (i.e. rainbow trout) contrasts with the apparent absence of this role for CA in the kidney of marine fish (Hodler et al., 1955; Swenson and Maren, 1986; Maren et al., 1992; Swenson, 2003). Particularly striking is the marine elasmobranch (*Squalus acanthias*) that has long been thought to lack renal CA but nevertheless displays a remarkable capacity to reabsorb  $\text{HCO}_3^-$  from the renal filtrate. Indeed, it has been documented that even during periods of metabolic alkalosis when plasma  $\text{HCO}_3^-$  levels increase to  $>30\text{mmol l}^{-1}$ , urinary loss of  $\text{HCO}_3^-$  remains minimal and constant (Swenson and Maren, 1986). In both dogfish (Swenson and Maren, 1986) and longhorned sculpin (Hodler et al., 1955; Maren et al., 1992), CA inhibition is without effect on urine acid-base status. In the light of these observations and additional *in situ* measurements of renal acidification in the marine skate *Raja erinacea*, a novel mechanism of  $\text{HCO}_3^-$  reabsorption was proposed for elasmobranchs involving

the direct transfer of ionic  $\text{HCO}_3^-$  from filtrate to lumen (Deetjen and Maren, 1974). Years later, Swenson and colleagues (Swenson et al., 1994) proposed a mechanism for CA-independent urinary acidification involving the secretion of  $\text{H}^+$  via a luminal  $\text{H}^+, \text{K}^+$ -ATPase. Although these findings led to, or supported, acceptance of the hypothesis that marine fish lack renal CA, this conclusion is no longer believed given the physiological evidence for cytosolic CA activity in proximal tubules of winter flounder (Renfro et al., 1999; Pelis et al., 2003; Pelis and Renfro, 2004) and the finding of membrane-associated CA IV mRNA in dogfish kidney (Gilmour et al., 2007a). While playing a role in renal  $\text{SO}_4^{2-}$  excretion, the potential involvement of CA in renal acidification and  $\text{HCO}_3^-$  reabsorption may be constrained by other factors such as the absence of suitable ion transporters (e.g. NHE or V-ATPase) on the luminal membrane. Considering that only a few (three) species have been examined and given the claims of radical differences in the involvement of renal CA in freshwater and marine fish, we encourage a re-examination of the involvement of CA in urinary acidification and  $\text{HCO}_3^-$  reabsorption in marine fish species.

#### CA in piscine-specific acid–base regulatory mechanisms

Thus far, this review has focused on the role of CA in the key tissues (gill and kidney) that contribute to the regulation of systemic acid–base status. Additionally, however, CA has been implicated in several other structures exhibiting physiological properties that are unique to certain fish. These include secretion of NaCl by the elasmobranch rectal gland, secretion of  $\text{H}^+$  by the gas gland of the teleost swimbladder, acidification by the pseudobranch of the arterial blood supplying the eye, and the addition of  $\text{HCO}_3^-$  to gut fluid by intestinal epithelial cells [please refer to the review by Grosell et al. in this issue (Grosell et al., 2009)].

##### Elasmobranch rectal gland

The rectal gland of elasmobranchs is a sausage-shaped structure that is capable of secreting into the posterior intestine a solution of roughly  $500 \text{ mmol l}^{-1}$  NaCl (Burger and Hess, 1960). The volume of rectal gland secretion produced is highly variable but is clearly increased by blood volume expansion associated with feeding/ingestion of seawater (Evans, 1993). While it has long been held that the rectal gland plays an important role in extra-renal salt excretion (reviewed by Shuttleworth, 1988), studies that have physically removed or ablated the gland have documented relatively minor (Evans et al., 1982; Evans, 1993) or no (Chan et al., 1967; Wilson et al., 2002; Wilson and Laurent, 2002) significant impact on plasma ion levels. Obviously, therefore, other routes of net salt excretion exist in elasmobranchs. The two sites that first spring to mind are the kidney and the gill. As discussed by Evans et al. (Evans et al., 2005), because marine elasmobranchs are slightly hyperosmotic, they gain water by osmosis that is balanced by equivalent volumes of urine production, thereby providing a route of salt efflux normally not present in most marine species. Although the gill is generally discounted as a site of net NaCl excretion in elasmobranchs (Shuttleworth, 1988), its potential involvement in extrarenal salt excretion should probably be reconsidered.

CA is abundant in the rectal gland (Lacy, 1983) where it plays an important role in fluid secretion; *in vivo* administration of CA inhibitors markedly reduced the rates of glandular secretion in dogfish (Swenson and Maren, 1984; Shuttleworth et al., 2006). It has been suggested that the role of CA in rectal gland secretion is to facilitate removal of metabolically produced  $\text{CO}_2$  so as to maintain an appropriate intracellular acid–base status (Swenson

and Maren, 1984). Two mechanisms of CA action have been proposed; Swenson and Maren (Swenson and Maren, 1984) suggested that cytosolic CA catalyses the inter-conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  while Shuttleworth and colleagues (Shuttleworth et al., 2006) concluded that a more likely scenario was the catalysed hydration of  $\text{CO}_2$  by membrane-associated CA in the extracellular compartment. In both models, CA contributes to  $\text{CO}_2$  removal from cells by enhancing  $\text{CO}_2$  diffusion gradients. Future studies should incorporate specific permeant and impermeant inhibitors to differentiate the potential roles of cytosolic *versus* extracellular (membrane-associated) CA.

Rectal gland fluid output is directly related to blood pH, rising with alkalosis and falling with acidosis (Swenson and Maren, 1984; Shuttleworth et al., 2006; Wood et al., 2007). Because blood pH in dogfish increases after eating [alkaline tide (Wood et al., 2005)], there would be an associated post-prandial increase in rectal gland secretion to aid in the excretion of the additional salt and water gained during ingestion. Similarly, a reduction in blood pH associated with exhaustive exercise would act as a cue to inhibit glandular secretion and thus help to retain fluid during periods of increased fluid entry into muscle (Shuttleworth et al., 2006; Wood et al., 2007).

##### The swimbladder

Inflation of the swimbladder in deep sea fish relies on the secretion of  $\text{O}_2$  to generate exceedingly high partial pressures of  $\text{O}_2$  (Pelster, 2004). The  $\text{O}_2$  is derived from haemoglobin *via* the Root effect following acidification of the blood by the swimbladder gas gland. In addition to  $\text{CO}_2$ , a variety of acidic metabolites contribute to the acidification of the blood in the gas gland including lactic acid and  $\text{H}^+$  originating from  $\text{Na}^+/\text{H}^+$  exchange and V-type  $\text{H}^+$ -ATPase activities (Pelster, 1995). A role for CA in gas gland acid secretion was first suggested by the results of experiments showing that gas deposition was slowed by CA inhibition (Fange, 1953). Subsequent experiments have shown that CA inhibition reduces lactate release by eel swimbladder *in vitro* (Kutchai, 1971) or acid secretion from isolated gas gland cells (Pelster, 1995). Because acid secretion from isolated gas gland cells was inhibited by membrane-permeant and -impermeant inhibitors, it was proposed that membrane-associated (extracellular) CA IV was involved (Pelster, 1995). The existence of membrane-associated CA (proposed to be CA IV) on the blood-facing plasma membranes and capillary endothelium was later confirmed using histochemistry (Wurtz et al., 1999). Future research should evaluate CA isoforms as well as the relative involvement of membrane-associated and cytosolic CA in swimbladder inflation.

##### The pseudobranch

Although the physiological function of the pseudobranch has long been debated (Laurent and Rouzeau, 1972; Laurent and Dunel-Erb, 1984; Bridges et al., 1998), there is accumulating evidence that one of its roles is to assist the  $\text{O}_2$ -concentrating mechanisms occurring within the choroid rete of the eye of certain marine teleosts. In these fish, the retina is thick and non-vascularized, thereby impeding the delivery of  $\text{O}_2$  to the retinal tissue. To meet the high metabolic demand of the retinal pigmented epithelium, extremely high partial pressures of  $\text{O}_2$  are generated (reported to exceed 1 atm,  $\sim 101 \text{ kPa}$ ) to facilitate diffusion. As in the swimbladder (Pelster, 2004), the mechanism thought to underlie the  $\text{O}_2$ -concentrating mechanism is the Root effect (for reviews, see Brittain, 1987; Pelster and Weber, 1991; Berenbrink, 2007) whereby acidification of the blood entering the rete causes  $\text{O}_2$  to be unloaded from haemoglobin for

delivery to the eye. The most compelling empirical evidence in support of a role for the Root effect is the observation that O<sub>2</sub> secretion into the ocular fluid of an *in vitro* blood-perfused rainbow trout eye preparation occurs only when blood exhibiting a Root effect is used as the perfusion fluid (i.e. trout, but not human blood) (Waser and Heisler, 2005). Although blood can be acidified in the choroid rete as well as in the eye itself, it has been proposed that the pseudobranch plays a crucial role in the pre-conditioning of blood prior to its entry into the rete (Bridges et al., 1998). The addition of CO<sub>2</sub> and H<sup>+</sup> to the blood flowing within the pseudobranch is thought to acidify the RBCs so as to approach the threshold for the onset of the Root effect. Such pre-conditioning would allow maximal release of O<sub>2</sub> from haemoglobin with minimal acidification (Bridges et al., 1998). A minimally acidified environment is important because unlike the swimbladder, which can tolerate extreme acidification (pH 6.5), the retinal tissue is intolerant of comparable reductions in pH. The mechanism of acidification of the blood within the pseudobranch involves the direct excretion of CO<sub>2</sub> and secretion of H<sup>+</sup> via Na<sup>+</sup>/H<sup>+</sup> exchange and V-type H<sup>+</sup>-ATPase (Kern et al., 2002). The importance of CA in sustaining these acidifying processes and allowing O<sub>2</sub> concentration within the eye was convincingly demonstrated (Fairbanks et al., 1969; Fairbanks et al., 1974) although to date there are no data on the identification of the participating isoforms. It also should be recognized that the pseudobranch is but one of several sites participating in CA-mediated acidification of pre-ocular blood.

### Conclusions

The above discussion emphasizes the point that the diversity of CA isoforms and functions in fish is at least as great as that in tetrapods, if not more so owing to the variation among fish species and groups, yet our knowledge of CA isoform diversity, distribution, function and regulation in fish lags well behind that for mammals. Particularly critical is a need for more genomic information that can be used to identify CA isoforms and clarify the evolutionary relationships with respect to CA between fish and tetrapods. Better tools, including fish species- and isoform-specific CA antibodies, are required to describe the cellular localization of CA in the tissues, gill and kidney, that are primarily responsible for acid-base regulation in fish. Physiological data are needed to speak to the functional significance of CA in acid-base processes. Finally, the regulation of CA expression and activity warrant investigation, together with the potential regulatory roles of CA. Accomplishing these goals for even a single fish species is a challenge but one that needs to be addressed if an integrated picture of the role of CA in acid-base regulation in fish is to be achieved.

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