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Acid–base balance and CO_2 excretion in fish: Unanswered questions and emerging models☆

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Abstract

Carbon dioxide (CO_2) excretion and acid–base regulation in fish are linked, as in other animals, though the reversible reactions of CO_2 and the acid–base equivalents H^+ and HCO_3^-: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. These relationships offer two potential routes through which acid–base disturbances may be regulated. Respiratory compensation involves manipulation of ventilation so as to retain CO_2 or enhance CO_2 loss, with the concomitant readjustment of the CO_2 reaction equilibrium and the resultant changes in H^+ levels. In metabolic compensation, rates of direct H^+ and HCO_3^- exchange with the environment are manipulated to achieve the required regulation of pH; in this case, hydration of CO_2 yields the necessary H^+ and HCO_3^- for exchange. Because ventilation in fish is keyed primarily to the demands of extracting O_2 from a medium of low O_2 content, the capacity to utilize respiratory compensation of acid–base disturbances is limited and metabolic compensation across the gill is the primary mechanism for re-establishing pH balance. The contribution of branchial acid–base exchanges to pH compensation is widely recognized, but the molecular mechanisms underlying these exchanges remain unclear. The relatively recent application of molecular approaches to this question is generating data, sometimes conflicting, from which models of branchial acid–base exchange are gradually emerging. The critical importance of the gill in acid–base compensation in fish, however, has made it easy to overlook other potential contributors. Recently, attention has been focused on the role of the kidney and particularly the molecular mechanisms responsible for HCO_3^- reabsorption. It is becoming apparent that, at least in freshwater fish, the responses of the kidney are both flexible and essential to complement the role of the gill in metabolic compensation. Finally, while respiratory compensation in fish is usually discounted, the few studies that have thoroughly characterized ventilatory responses during acid–base disturbances in fish suggest that breathing may, in fact, be adjusted in response to pH imbalances. How this is accomplished and the role it plays in re-establishing acid–base balance are questions that remain to be answered.

Keywords: CO_2 excretion; Acid–base balance; Carbonic anhydrase; Fish; Gill; Kidney; Ventilation; Chemoreceptors; NHE; NBC; H^+-ATPase

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1. Introduction

Carbon dioxide (CO_2) excretion and acid–base regulation are inextricably linked in fish, as in other animals, through the reversible hydration/dehydration reactions of CO_2 and the acid–base equivalents H^+ and HCO_3^-: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. These reactions are catalyzed by the enzyme carbonic anhydrase (CA), which therefore has key roles to play in both CO_2 excretion and acid–base regulation (reviewed by Perry and Laurent (1990), Randall and Val (1995), Henry and Heming (1998), Geers and Gros (2000), Swenson (2000) and Henry and Swenson (2000)). Metabolic CO_2 production would, in the absence of CO_2 elimination, result in the toxic accumulation of protons (respiratory acidosis). Indeed, environmental and/or physiological conditions that lower ventilation, such as exposure to hyperoxia, typically elicit a respiratory acidosis owing to CO_2 retention, whereas conditions promoting hyperventilation may be accompanied by a respiratory alkalosis as CO_2 transfer is enhanced (see Table 1 in Gilmour (2001)). The flip side of this coin is that the potential exists to regulate metabolic acid–base disturbances through the control of ventilation. This strategy figures prominently in tetrapods where, for example, increasing pulmonary ventilation constitutes a key response to metabolic acidosis that serves to reduce the arterial partial pressure of CO_2 (Pa_{CO_2}), thereby raising arterial pH (pHa) (Heisler, 1986; Swenson, 2000). The success of this strategy relies on the relatively high initial Pa_{CO_2} (e.g. ~40 Torr in humans), however, and because Pa_{CO_2} values of ~2 Torr are typical of water-breathing fish, the capacity of most fish to use hyperventilatory responses to manipulate acid–base status is limited (Heisler, 1986; Claiborne et al., 2002). Generally low Pa_{CO_2} values in fish reflect several factors; primarily the impact on CO_2 transfer of the high convection requirements for O_2 uptake (ventilation volume per unit O_2 uptake) in a medium of relatively low O_2 content, and the countercurrent arrangement of blood flow through the gills and water flow across the gills. Under these conditions, hypoventilatory adjustments for the purpose of correcting acid–base disturbances clearly will not be favoured owing to the likely negative impact on O_2 delivery.

With respiratory compensation being of limited importance, the linchpin of acid–base regulation in most fish is the direct transfer of acid–base equivalents between the animal and the external environment, primarily across the gills (for recent reviews, see Goss et al., 1998; Claiborne et al., 2002; Perry et al., 2003b; Evans et al., 2005). Specifically, pH compensation is achieved by adjusting plasma HCO_3^- levels through the differential regulation of H^+ and HCO_3^- effluxes which are widely believed to be coupled to, respectively, the influx of Na^+ and Cl^-. Yet despite decades of study, the cellular and molecular mechanisms underlying these branchial transfers remain unclear, as do the proximate pathways through which adjustments of H^+ and HCO_3^- effluxes are initiated. The quantitative importance of the gill in acid–base compensation in fish – it is thought to account for 90% or more of acid–base movements in most species (Claiborne et al., 2002) – has made it easy to overlook other potential contributors. Acid–base transfer occurs across the intestinal epithelium of seawater fish, but does not appear to be dynamically regulated to contribute to the control of acid–base status (Wilson et al., 1996, 2002). Recent work has, however, emphasized the critical involvement of the kidney in acid–base regulation in fish (Wood et al., 1999; Georgalis et al., 2006a; see reviews by Perry and Fryer (1997) and Perry et al. (2003b)), although again, little information is available on the mechanisms of urine acidification in fish at the molecular level. Given this background, and keeping in mind the many comprehensive reviews of various aspects of gas transfer and acid–base regulation available in the recent literature (e.g. Henry and Swenson, 2000; Gilmour, 2001; Perry and Gilmour, 2002; Claiborne et al., 2002; Evans et al., 2005), the present paper aims to highlight unanswered questions about pH balance and CO_2 excretion in fish, while at the same time outlining emerging models of acid–base regulation.

2. CO_2 transfer and acid–base regulation

Ventilatory adjustments for the purpose of acid–base regulation are initiated in tetrapod vertebrates by the activation of peripheral and central chemoreceptors sensitive to CO_2 and/or pH (e.g. Gonzalez et al., 1994; Milsom, 1995, 2002; Nattie, 1999; Taylor et al., 1999). Whether equivalent chemoreceptors exist in fish remains a topic of some debate. The existence of a CO_2-keyed ventilatory drive in fish is now well established (reviewed by Milsom (1995, 2002), Gilmour (2001)

and Gilmour and Perry (in press)), but the characteristics of the chemoreceptors responsible for this drive are less clear. Apart from some data for bimodally breathing species (Wilson et al., 2000b; Gilmour, 2001; Sanchez et al., 2001; Remmers et al., 2001), there is little experimental support for the existence of central CO_2 chemosensitivity in fish (Milsom, 1995, 2002; Gilmour, 2001; Gilmour and Perry, in press). Rather, CO_2-initiated ventilatory reflexes appear to be dominated by branchial chemoreceptors (Burleson and Smatresk, 2000; Reid et al., 2000; Sundin et al., 2000; McKendry et al., 2001; Perry and Reid, 2002) that are sensitive primarily to changes in the CO_2 tension of water flowing over the gills, not those in blood perfusing the gills (McKendry and Perry, 2001; Perry and McKendry, 2001; Perry and Reid, 2002; Gilmour et al., 2005). The available evidence additionally suggests that CO_2, rather than any accompanying change in water pH, is the proximate stimulus for receptor activation (Reid et al., 2000; Sundin et al., 2000; Perry and McKendry, 2001; Gilmour et al., 2005), although by analogy with mammalian chemoreceptors, the transduction of CO_2 stimuli likely involves the intracellular conversion, catalyzed by CA, of CO_2 to H^+ (Lahiri and Forster, 2003; Putnam et al., 2004). The cellular identification of branchial CO_2 chemoreceptors and elucidation of the chemotransduction mechanism(s) involved in CO_2 sensing are obvious gaps in our knowledge that need to be addressed.

Despite the apparently exclusively external (water-sensing) orientation of branchial CO_2-sensitive chemoreceptors, and the theoretical constraints outlined above on the contribution of ventilatory adjustments to the modulation of acid–base status in an animal of low Pa_{CO_2}, some evidence suggests that fish not only respond to internal P_{CO_2}/pH levels, but in fact alter ventilation in a fashion appropriate to correct imbalances in these levels (reviewed by Gilmour (2001)). In rainbow trout (*Oncorhynchus mykiss*) violently exercised to exhaustion, the early stages of recovery are marked by a significant acidosis of combined metabolic and respiratory origin. Metabolic protons are added to the blood from anaerobic metabolism in the white muscle, and as a result of the adrenergic activation of the red blood cell (RBC) Na^+–H^+ exchanger (β-NHE; Borgese et al., 1992, 1994). The likely sources of the 50–400% rise in Pa_{CO_2} (Wood and Perry, 1985) include titration of plasma HCO_3^- ions to CO_2 by metabolic protons, the inhibition of RBC CO_2 excretion by catecholamines (reviewed by Tufts and Perry (1998)), and the effects of increased cardiac output on branchial CO_2 transfer (CO_2 transfer behaves as a diffusion-limited system, at least in teleost fish; reviewed by Perry and Gilmour (2002)). The post-exercise acidosis is accompanied by a hyperventilatory response that may aid in replenishing O_2 stores depleted during exhaustive exercise (the O_2 debt; Wood and Perry, 1985; Perry and Wood, 1989; Tufts and Perry, 1998), but that clearly also must serve to correct the acidosis itself, a function that largely has been overlooked. The post-exercise hyperventilation in rainbow trout appears to be keyed to the acidosis, because the hyperventilatory response was reduced by treatment of rainbow trout with bovine CA to enhance CO_2 excretion and hence attenuate the magnitude of the acidosis (Wood and Munger, 1994). This finding argues convincingly in favour of the presence of chemoreceptors that monitor blood CO_2/pH levels. Similar conclusions can be drawn from the analyses of ventilation and blood acid–base status in hyperoxic dogfish (*Scyliorhinus* sp.) carried out by Heisler and co-workers (Heisler, 1988; Heisler et al., 1988). Exposure of dogfish to environmental hyperoxia caused an immediate and profound hypoventilation associated with a rapid increase in Pa_{CO_2} (Heisler et al., 1988). The rate of increase of Pa_{CO_2} was dampened, however, by a transient relative hyperventilation that Heisler et al. (1988) hypothesized was a response to the rapid rise in Pa_{CO_2} and/or concomitant fall in pHa (respiratory acidosis) to restore acid–base status. The reduction in Pa_{CO_2} reported for hyperoxic rainbow trout in response to experimentally induced hyperventilation attests to the potential contribution of ventilatory adjustments to acid–base status under these conditions (Wood and Jackson, 1980). Again, however, such an adjustment of ventilation implies the capacity to monitor internal P_{CO_2} and/or pH status. Heisler and colleagues (unpublished data reported in Heisler, 1988) supported this possibility by documenting strong correlations between pHa and changes in ventilation in hyperoxic dogfish in which arterial acid–base status was manipulated by varying the Pa_{CO_2} of inspired water and/or infusing base loads.

Countering the above studies that suggest that ventilation in fish may be influenced by blood acid–base status for the purpose of correcting acid–base disturbances is a number of studies in which no impact

Table 1
Data from selected experimental studies on the effects of acid or base infusion on arterial acid–base status and ventilation

Species	Base/acid load (μmol kg^{-1})	ΔpHa	ΔPa_{CO_2} (Torr)	$\Delta \dot{V}_w$ (%)	Δf_R (%)	ΔV_{amp} (%)	Reference
Base							
Rainbow trout, *O. mykiss*	4.1 $NaHCO_3$, injection	0.32†		189†			Janssen and Randall (1975)
	250 NaOH over 10 min	0.26*	0.7*	75*			McKenzie et al. (1993)
	1000 $NaHCO_3$ over 10 min	0.13*	0.2		10	140*	McKenzie et al. (1993)
	2000 $NaHCO_3$ over 10 min	0.30*	1.18*		3	1	Gilmour and Perry (1996)
Spiny dogfish, *Squalus acanthias*	4800 $NaHCO_3$ over 3 h	0.256*	0.37*		−3	28*	Gilmour et al. (2001)
Acid							
Rainbow trout, *O. mykiss*	94.7 HCl, injection	−0.20†		276†	21		Janssen and Randall (1975)
	250 HCl over 10 min	−0.15*	0.7	85*	5	140*	McKenzie et al. (1993)
	200 HCl over 6 min	−0.26*			4	183*	Aota et al. (1990)
	400 $(NH_4)_2SO_4$ over 10 min	−0.21*	−0.04		3	28*	Gilmour and Perry (1996)
Bowfin, *Amia calva*	250 HCl over 8.5 min	−0.29*	6.49*		40 (1256*)[a]	85*	McKenzie et al. (1991)

Changes in ventilation volume ($\Delta \dot{V}_w$), or ventilation frequency (Δf_R) and ventilation amplitude (ΔV_{amp}), are expressed as a percentage of the control value together with changes in arterial pH (ΔpHa) and arterial P_{CO_2} (ΔPa_{CO_2}); thus a negative value indicates a decrease while a positive value indicates an increase from the control value. Percent changes have been calculated from mean data reported in the original studies. Where time course data were presented in the original study, peak changes have been calculated. An asterisk (*) indicates a significant effect of the acid or base load, whereas a dagger (†) indicates a difference that appeared to be significant but was not tested statistically tested in the original study. Empty cells indicate that a particular parameter was not measured in that study.

[a] The percent increase in the frequency of air breathing.

of internal acid–base disturbances on ventilation has been detected. In particular, a variety of experimental approaches have been used to induce a respiratory acidosis, including a reduction in gill surface area (Julio et al., 2000), an increase in the blood-to-water diffusion distance (Bindon et al., 1994) or the inhibition of RBC CA activity with acetazolamide (see Table 3 in Gilmour (2001), as well as Gilmour et al. (2005)), all of which serve to impair CO_2 excretion. In each case, however, the resultant respiratory acidosis failed to elicit corrective changes in ventilation.

Similarly mixed results have been reported for the effects of metabolic acid–base disturbances on ventilation (Table 1). Whereas acid loads have generally elicited the expected increases in ventilation, hyperventilatory responses also generally accompanied base loads. However, scrutiny of studies in which ventilation has been measured in fish subjected to metabolic acid or base loads reveals a number of difficulties in terms of interpreting changes in ventilation as responses to acid–base disturbances. First, increases in Pa_{CO_2} in base-infused fish may confound the effects of a rise in pHa on ventilation (Table 1). Blood O_2 status and/or circulating catecholamine concentrations were affected by acid or base infusion in some studies, and not measured in others; again, these may act as confounding factors (Randall and Taylor, 1991; Perry et al., 1992). Perhaps most importantly, however, in most cases the primary goal of the study was not to determine whether ventilatory changes contribute to the correction of metabolic acid–base disturbances, and hence the experimental design was less than ideal for this purpose. In particular, the time course of the acid–base disturbance induced in most studies was quite brief (minutes rather

than hours). Given the apparent capacity of a metabolic acidosis to induce hyperventilation (Table 1), a more detailed assessment of the contribution of ventilatory adjustments to the correction of metabolic acid–base disturbances appears to be warranted.

Fish that breathe both air and water (bimodal breathers) provide insight into the conflict in ventilatory requirements between O_2 delivery and acid–base regulation faced by unimodal water breathers. Although bimodal breathers vary in their reliance on air breathing, it is widely accepted that air breathing is used to satisfy requirements for O_2 uptake while the gills (and/or skin) remain the primary site for CO_2 excretion (Burggren and Johansen, 1986). In some species (Perry et al., 2005) or under conditions of increased metabolic rate (Amin-Naves et al., 2004), however, aerial CO_2 transfer may become as or more important than aquatic CO_2 transfer. As the reliance on air-breathing increases, typically the functional surface area of the gills is reduced and the blood-to-water diffusion distance increases (e.g. Brauner et al., 2004a), with a consequent increase in Pa_{CO_2}. The higher Pa_{CO_2} coupled with the capacity to independently modulate O_2 uptake and CO_2 excretion suggest that these species should make greater use of ventilatory adjustments for the regulation of acid–base status. Moreover, some evidence suggests that bimodally breathing fish, like tetrapods, possess central CO_2/pH chemosensitivity that contributes to the regulation of air breathing (Wilson et al., 2000b; Gilmour, 2001; Sanchez et al., 2001; Remmers et al., 2001; Gilmour and Perry, in press). To date, however, little information is available on the regulation of acid–base disturbances in bimodal breathers and the contribution of ventilatory adjustments to this process. Burleson et al. (1998) assessed ventilation in spotted gar (*Lepisosteus oculatus*) during recovery from the acidosis incurred as a result of exhaustive exercise. Although spotted gar increased branchial ventilation post-exercise ($V_{amp} \sim 63\%$, f_R not significant), air-breathing appeared to increase to a much greater extent (~293% increase in air-breathing frequency), and Burleson et al. (1998) attributed the prolonged recovery of gar (4–8 h versus 1–2 h in water-breathing fish; Wood and Perry, 1985) from the post-exercise respiratory acidosis to the reduced role of branchial ventilation in this species. In support of this argument, further reductions in branchial ventilation achieved by exposing the fish to hypoxic water elicited a more profound respiratory acidosis (Burleson et al., 1998). Similarly, bowfin (*Amia calva*) forced to rely exclusively on branchial ventilation recovered more quickly from an exercise-induced acidosis than did those provided with access to air (Gonzalez et al., 2001). Recently, ventilatory responses to metabolic acid–base disturbances were examined in African lungfish (*Protopterus annectens*), an obligate air-breather with highly reduced gills (S.F. Perry, R. Euvermann, S.F. Chew, Y.K. Ip, K.M. Gilmour, unpublished data). Infusion of $NaHCO_3$ over a 1 h period to increase pHa significantly by 0.22 pH units in the absence of any change in Pa_{CO_2} resulted in a significant fall in CO_2 excretion into the water without affecting aerial CO_2 excretion (Fig. 1A). The reduction in aquatic CO_2 transfer, an appropriate response to correct the metabolic alkalosis, likely reflected decreased water flow across the gills as the frequency of opercular movements declined by, on average, 71% following base infusion. A separate group of lungfish was subjected to a 1 h infusion of NH_4Cl, which decreased pH by 0.17 pH units while at the same time increasing Pa_{CO_2} by almost 3 Torr. In response to this mixed metabolic/respiratory acidosis, transient increases in both aerial and aquatic CO_2 transfer occurred (Fig. 1B), reflecting increases of 183% and 256% in the frequency of air and water breathing, respectively. Thus, lungfish appear capable of detecting internal pH/CO_2 status and adjusting branchial and/or pulmonary ventilation appropriately for the correction of acid–base disturbances.

Clearly, whether or under what conditions ventilation is modified in fish by changes in blood pH/CO_2 status alone still remains uncertain, despite numerous studies. The uncertainty reflects in part the experimental challenges associated with manipulating internal CO_2 and pH status independently and without effect on blood O_2 levels (see Perry and Wood, 1989; Gilmour, 2001). Intra- and interspecific variation in ventilatory responses also contribute to the complexity of the situation. It is not clear, for example, why an approximately 3 Torr increase in arterial P_{CO_2} following exhaustive exercise in rainbow trout is associated with a hyperventilatory response (Wood and Munger, 1994), but a comparable increase in arterial P_{CO_2} in trout treated with acetazolamide is without significant effect on ventilation (Gilmour et al., 2001). Finally, species vary in their tolerance of pH imbalance (e.g. see Brauner et al., 2004b) as well as in the form and function of avail-

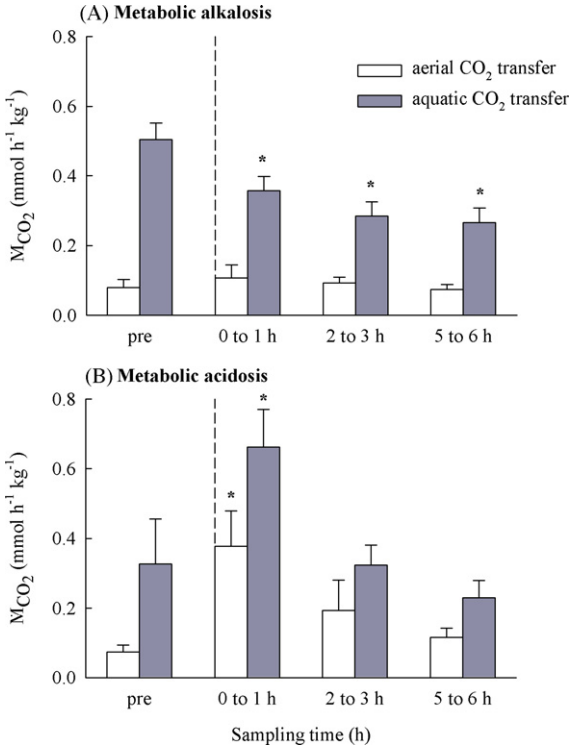

Fig. 1. The effects on CO_2 excretion ($\dot{M}_{CO_2}$) in *Protopterus annectens* of the infusion of (A) $NaHCO_3$ ($N = 8$) and (B) NH_4Cl ($N = 6$–9). Absolute rates of CO_2 excretion derived from changes in the partial pressure of CO_2 in the air (unfilled bars) and water (filled bars) were determined using a customized respirometer with both air and water chambers as described by Perry et al. (2005) for 1 h periods prior to, during, and at 2 and 5 h following base or acid infusion. The initiation of the 1 h base or acid infusion period is denoted by the dotted line. Values are mean ± 1 S.E.M. Asterisk (*) indicates means that are significantly different from the corresponding "pre" value [one-way repeated measures analysis of variance, $P \leq 0.001$ for aquatic $\dot{M}_{CO_2}$ in (A); $P \leq 0.001$ and 0.03 for, respectively, aquatic and aerial $\dot{M}_{CO_2}$ in (B)].

able gas exchange sites, and compensatory ventilatory responses to acid–base disturbances might therefore be expected to exhibit corresponding variation.

3. Branchial acid–base regulation

Although questions remain about the potential contribution of respiratory compensation to the correction of acid–base disturbances, the gill in water breathers is unquestionably the predominant site of metabolic compensation (see reviews by Goss et al. (1992b), Evans et al. (1999, 2005), Evans (2002), Marshall (2002), Claiborne et al. (2002), Hirose et al. (2003) and Perry et al. (2003b)). As noted above, the metabolic compensation is achieved by adjustments of the rates of transfer of acidic and basic equivalents between the internal and external compartments. Thus, during periods of acidosis, net acid efflux across the gill is increased (equivalent to reduced base efflux) whereas during alkalosis, the net efflux of acid across the gill is decreased (equivalent to increased base efflux), and accordingly, plasma HCO_3^- levels are increased during compensation of acidosis and decreased during compensation of alkalosis. Branchial acid–base regulation is intimately linked to ionic regulation owing to obligate relationships between the excretion of acidic and basic equivalents and the absorption of Na^+ and Cl^- from the water, a principle that applies to both freshwater (FW) and seawater (SW) species. Consequently, the pH and ion composition of the water in which the fish is living may have a profound impact on its ability to regulate acid–base disturbances via branchial mechanisms (e.g. Iwama and Heisler, 1991; Larsen and Jensen, 1997; Wood et al., 1998; Choe and Evans, 2003). While it is known that the excretion of H^+ is linked to uptake of Na^+ and that excretion HCO_3^- is related to uptake of Cl^-, the specific mechanisms underlying these relationships are not well understood. In recent years, however, the application of molecular techniques to this question has resulted in substantial advances in our understanding of the links between ionic uptake and acid–base regulation. These recent developments will be reviewed as a prelude to the proposal of integrated models of branchial acid–base regulation in fish.

3.1. Gill epithelial cell types and nomenclature

The gill epithelial cells that have been implicated in acid–base regulation are the mitochondria rich cells (MRCs; also termed chloride cells or ionocytes) and pavement cells (PVCs; also termed respiratory cells). In recent years these cell types have been further subclassified, at least in rainbow trout, largely on the basis of morphological differences (Goss et al., 2001; Galvez et al., 2002). For example, two distinct populations of MRCs exist; those exhibiting peanut lectin agglutinin (PNA) binding sites on their apical membranes (PNA^+ MRCs) and those lacking PNA binding

sites (PNA^- MRCs). Both cell types are enriched in Na^+/K^+-ATPase, but only the PNA^+ cells exhibit the extensive three-dimensional tubular network of basolateral membranes that typifies the classical chloride cell (Perry, 1997). It is likely that the PNA^- MRCs correspond to the mitochondria rich PVCs that were first described by Goss et al. (1992a, 1994a) in brown bullhead (*Ictalurus nebulosus*).

3.2. Links between Na^+ uptake and H^+ excretion

Currently, two potentially mutually inclusive mechanisms are thought to link Na^+ uptake and H^+ excretion. The classical model first proposed by Krogh (1938) incorporates an apical membrane electroneutral Na^+/H^+ exchanger (NHE). The NHEs comprise the SLC9A multi-gene family consisting of at least nine paralogs (NHE1–9; Brett et al., 2005). These exchangers move Na^+ and H^+ according to existing transmembrane chemical gradients, and it is for this reason that their role in Na^+ uptake in FW fish was first questioned (Avella and Bornancin, 1989). Indeed, it has been argued that the operation of such an apical membrane NHE is not feasible in FW owing to thermodynamic constraints. Branchial intracellular Na^+ concentration are estimated to be 55–80 mmol L^{-1} (see Table 1 in Morgan et al., 1994), whereas FW typically contains levels of Na^+ below 1 mmol L^{-1}. Thus, for NHE to function in an influx mode, an intracellular pH of approximately 6.5 (assuming external pH of 8.0) would be needed to overcome the unfavourable Na^+ gradient; so acidic an intracellular environment is likely incompatible with cell function. In SW fish, however, the inwardly directed Na^+ gradient would obviously favour the functioning of an apical membrane NHE. Indeed, there is now ample evidence for the existence of several NHE isoforms (NHEs1–3) in the gills of numerous SW species including agnathans (Edwards et al., 2001; Choe et al., 2002), elasmobranchs (Edwards et al., 2002; Choe et al., 2002, 2005; Tresguerres et al., 2005) and teleosts (Claiborne et al., 1999; Edwards et al., 1999, 2005; Scott et al., 2005). Interestingly, various NHE isoforms have also been localised to the gills of several species inhabiting FW (Edwards et al., 1999, 2005; Wilson et al., 2000a; Hirata et al., 2003; Scott et al., 2005; Choe et al., 2005). Moreover, NHE2 mRNA levels recently were reported to increase in killifish (*Fundulus heteroclitus*) after transfer from brackish water to FW (Scott et al., 2005) and in stingrays (*Dasyatis sabina*) transferred from SW to FW (Choe et al., 2005). Perhaps even more surprising is the localization of an NHE3 isoform to the apical membrane of MRCs in Osorezan dace (*Tribolodon hakonensis*) inhabiting highly acidic (pH 3.5) water (Hirata et al., 2003). In this case, the functioning of an apical NHE theoretically would be severely constrained by the combination of highly unfavourable Na^+ and H^+ gradients. Localised microenvironments (internal and external), in which Na^+ and H^+ levels might vary significantly from average intracellular and external values, may provide a possible explanation for the operation of apical membrane NHE in FW fish. For example, in PNA^+ MRCs (see above), the basolateral membrane is highly infolded and can therefore be in close proximity to the apical membrane. Thus, the pumping of Na^+ across the basolateral membrane via Na^+/K^+-ATPase may create pockets of low Na^+ concentration in the vicinity of apical membrane NHE. Alternatively, basolateral H^+ pumping via the V-type H^+-ATPase may create acidic microenvironments near apical membrane NHE. Such a scheme has been postulated to create a favourable inward electrochemical gradient for Na^+ entry through apical membrane Na^+ channels in FW killifish (Katoh et al., 2003) but might also serve to lower pH to assist electroneutral Na^+/H^+ exchange. Finally, an apical membrane Na^+/H^+ exchanger could be assisted by the presence of mucus on the external surface of the gill which has the capacity to concentrate Na^+ in the boundary layer of water adjacent to the apical membrane (Handy, 1989).

An alternate model linking Na^+ uptake and H^+ excretion is the presence of apical membrane V-type H^+-ATPase coupled energetically to apical membrane Na^+ channels (ENaC). According to this model, the export of H^+ into the water via the ATPase would help to hyperpolarize the plasma membrane, thereby creating a favourable electrochemical gradient for the inward diffusion of Na^+ via ENaC. This model (reviewed by Marshall (2002) and Evans et al. (2005)) is likely to be less applicable to SW fishes in which favourable gradients support ATP independent (passive) Na^+/H^+ exchange. Although substantial evidence exists for apical membrane H^+-ATPase in FW fishes (Lin et al., 1994; Lin and Randall, 1995; Sullivan et al., 1995; Perry, 1997; Perry and Fryer, 1997; Wilson et

al., 2000a), there are few data supporting the presence of ENaC. Wilson et al. (2000a) demonstrated apical membrane immunoreactivity in several teleost species using heterologous antibodies, but efforts to clone any of the four sub-units of this gene have so far proven unsuccessful. Moreover, searches of the zebrafish (*Danio rerio*) and pufferfish (*Fugu rubripes*) molecular databases suggest that ENaC is not in the genome of teleost fish. However, the results of two studies have provided experimental evidence for the H^+-ATPase-Na^+ uptake model. Fenwick et al. (1999) demonstrated that addition of bafilomycin, a V-type H^+-ATPase inhibitor, to the water inhibited Na^+ uptake in tilapia (*Oreochromis mossambiucs*) and carp (*Cyprinus carpio*). More recently, it was demonstrated in vitro that stimulation of H^+ efflux by PNA^- MRCs (see above) during intracellular acidosis was attenuated by the Na^+ channel inhibitor, phenamil (Reid et al., 2003). It is possible that Na^+ may be entering via another type of phenamil-sensitive monovalent cation-selective channel.

3.3. Links between Cl^- uptake and HCO_3^- excretion

Members of two multi-gene families, SLC26 (Mount and Romero, 2004) and SLC4 (Alper et al., 2002; Romero et al., 2004), have been implicated in mediating Cl^-/HCO_3^- exchange (anion exchange or AE) on the apical membrane of gill epithelial cells (see review by Evans et al. (2005)). Using heterologous antibodies (Wilson et al., 2000a) or oligonucleotide probes (Sullivan et al., 1996), an AE1-like (band 3-like) gene was identified in the apical region of MRCs. While these results support the existence of an apical Cl^-/HCO_3^- exchanger, the contention that AE1 specifically is involved should be treated with some caution. First, the possibility of cross-reactivity of the AE1 antibodies or probes with other anion exchangers cannot be ruled out. Second, there is no evidence for the presence of AE1 on the apical membrane of other acid/base secreting epithelia. Recently, a new member of the SLC4 gene family, AE4, was localised to the apical membrane of the base-secreting intercalated cells (β-intercalated cells) of the mammalian kidney (Tsuganezawa et al., 2001). It will be interesting to determine whether a similar protein exists on the apical membrane of fish gill MRCs.

The SLC26 gene family consists of at least ten members capable of exchanging a wide variety of monovalent and divalent anions including Cl^- and HCO_3^- (Mount and Romero, 2004). One member, SLC26A4 (pendrin), has been identified as a Cl^-/HCO_3^- exchanger on the apical membrane of β-intercalated cells of the mammalian distal tubule, where its expression is increased with metabolic alkalosis (Wagner et al., 2002). Recently, pendrin-like immunoreactivity was observed on the apical membrane of gill epithelial cells in FW Atlantic stingray (Piermarini et al., 2002); notably, these cells were enriched with basolateral V-type ATPase but not Na^+/K^+-ATPase. Similarly, basolateral membrane H^+-ATPase recently was immunolocalised to epithelial cells of the marine dogfish (*Squalus acanthias*) (Tresguerres et al., 2005) that also lacked Na^+/K^+-ATPase enrichment. The physiological significance of these findings is discussed below.

3.4. An integrated model for branchial acid–base regulation

By analogy to the mammalian kidney, we wish to propose a model for acid–base regulation at the fish gill in which PNA^- MRCs act as acid-secreting cells and PNA^+ cells function as base-secreting cells (Fig. 2). Because these cell types have only been described in FW rainbow trout, the applicability of such a model to other species is uncertain, although the proposed model incorporates elements of acid–base transport from both FW and SW fish.

As suggested previously (Galvez et al., 2002; Reid et al., 2003), the PNA^- MR cells, by analogy with the α-intercalated cells of the mammalian distal tubule, are proposed to act as acid-secreting cells while also being largely responsible for Na^+ uptake. Na^+ uptake is accomplished by a Na^+/H^+ exchanger (NHE2 or NHE3) and/or via a Na^+ channel (possibly ENaC) linked to a V-type H^+-ATPase. The involvement of NHE would be more likely in SW species, although in FW fish, the gradient for apical membrane Na^+/H^+ exchange could result from the export of intracellular Na^+ via the basolateral membrane Na^+/K^+-ATPase. Net acid excretion would be achieved by the combined actions of apical membrane H^+ efflux and basolateral membrane HCO_3^- efflux. The latter step could be accomplished by a Cl^-/HCO_3^- exchanger or Na^+-HCO_3^- co-transporter (Hirose et al., 2003; Perry et al.,

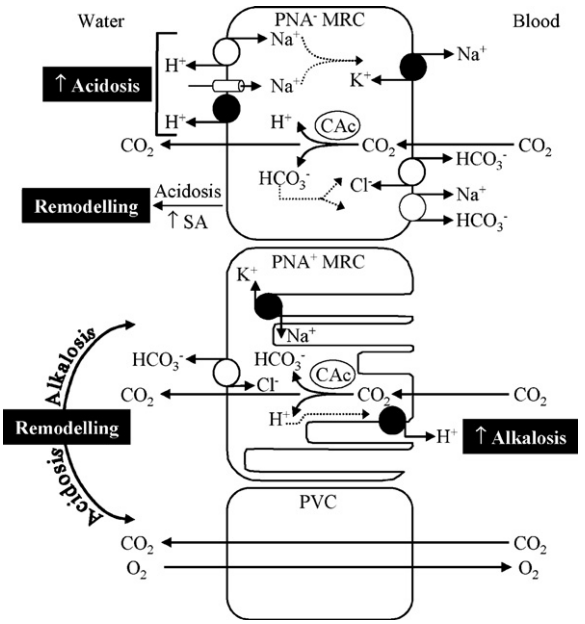

Fig. 2. A schematic depicting the proposed model for acid–base regulation in the rainbow trout gill. The PNA^+ mitochondria rich cells (MRCs) act as base-excreting cells by possessing apical membrane Cl^-/HCO_3^- exchangers and basolateral V-type H^+-ATPase. The PNA^- MRCs function as acid-excreting cells by possessing apical membrane Na^+/H^+ exchangers and/or Na^+ channels linked to a V-type H^+-ATPase, coupled with basolateral Cl^-/HCO_3^- exchangers or Na^+/HCO_3^- co-transporters. Transporters known to be transcriptionally activated by acid–base disturbances are indicated. In addition a scheme for regulating the rate of Cl^-/HCO_3^- exchange by PNA^+ MRCs is presented whereby adjacent pavements cells (PVCs) can expand or retract to alter the surface of the apical membrane exposed to the water. Similarly, experimental evidence exists to support the occurrence of changes in the exposed apical surface area (SA) of the PNA^- MRC cell type under acidotic conditions. Energy-consuming transporters are indicated by filled circles. CAc refers to cytosolic carbonic anhydrase. Note that whereas the figure depicts one cell of each type, the three cell types differ in abundance in the fish gill, with PVCs accounting for ~80% of all cells in the branchial epithelium, and PNA^+ and PNA^- MRCs each accounting for ~10% of all cells (Goss et al., 2001).

2003a). The cell type responsible for base excretion is proposed to be the PNA^+ MRC in which an apical membrane Cl^-/HCO_3^- exchanger links Cl^- uptake to HCO_3^- excretion. Apical membrane HCO_3^- efflux in concert with basolateral H^+ efflux (via a V-type H^+-ATPase) would accomplish net transepithelial base excretion. In both cell types, CA mediates the production of the substrates, HCO_3^- and H^+, via the catalysed hydration of molecular CO_2 (reviewed by Perry and Laurent (1990), Randall and Val (1995), Henry and Swenson (2000) and Hirose et al. (2003)). Although the branchial cytoplasmic CA has traditionally been termed CA isoform II (CA II), recent molecular evidence indicates that it is one of two cytoplasmic isoforms both of which are ancestral to the CA II of mammals or tetrapod vertebrates (Esbaugh et al., 2005). Recently, Georgalis et al. (2006b) provided direct evidence for a role for gill CA in regulating acid–base balance by demonstrating a pronounced reduction in branchial acid efflux in hypercapnic trout subjected to CA inhibition.

There is considerable evidence that the transporters/exchangers involved in acid–base regulation are transciptionally regulated during acid–base disturbances to modulate net acid flux across the gill (see Table 2). The mechanisms underlying these transcriptional changes are not yet known although the glucocorticoid stress hormone, cortisol, has been found to increase V-type H^+-ATPase activity in rainbow trout (Lin and Randall, 1993). In the mammalian kidney, glucocorticoids play an important role in regulating NHE activity (Hayashi et al., 2002) and thus may also play role in regulating branchial NHEs.

In addition to transcriptional regulation, there is the possibility that the rates of branchial acid–base equivalent fluxes can be regulated by gill remodelling during acid–base disturbances (Goss et al., 1992a, 1994a,b; Goss and Perry, 1993; Laurent et al., 1994). According to this model (reviewed by Goss et al. (1992b, 1995), Perry and Laurent (1993) and Laurent and Perry (1995)), net acid excretion across the gill can be controlled by regulating the surface area of the apical membranes of the acid- and base-excreting cells that is exposed to the water. The original model was formulated before MRC sub-types had been identified in the gill and thus it was assumed that MRCs (chloride cells) are the sites of base excretion and PVCs are the sites of acid excretion. The model has been refined (Fig. 2) assuming that PNA^+ MRCs are the base-excreting cells and that PNA^- MRCs are the acid-excreting cells. Thus, during acidosis, a marked reduction in the surface area of PNA^+ MRCs is proposed to occur, owing to their physical covering by PVCs. Concomitantly, there is an increase in the apical surface area of PNA^- cells. The net consequence of these changes is a reduction in the numbers of functional Cl^-/HCO_3^- exchangers (because of their presumed association with the

Table 2
Data from selected experimental studies on the effects of acid–base disturbances on branchial transporters of acid–base equivalents

	AE1-like	NHE1	NHE2	NHE3	NHE-like	V-type H^+-ATPase	Reference
Rainbow trout, *O. mykiss*, FW							
Metabolic alkalosis	↑ mRNA in MRCs						Sullivan et al. (1996)
Respiratory acidosis						↑ mRNA in PVCs	Sullivan et al. (1996)
Respiratory acidosis						↑ in PVCs	Sullivan et al. (1995)
Respiratory acidosis						↑ in PNA^- MRCs	Galvez et al. (2002)
Respiratory acidosis						↑ Activity	Lin and Randall (1993)
Sculpin, *Myoxocephalus octodecimspinosus*, SW, metabolic acidosis		↓ mRNA					Claiborne et al. (1999)
Killifish, *F. heteroclitus*							
FW, respiratory acidosis		NC	↑				Edwards et al. (2005)
SW, respiratory acidosis		↑		↑			Edwards et al. (2005)
Stingray, *D. sabina*, FW, respiratory acidosis			NC	NC			Choe et al. (2005)
Spiny dogfish, *S. acanthias*, SW							
Metabolic acidosis			↑				Tresguerres et al. (2005)
Metabolic alkalosis						↑ Basolateral expression	Tresguerres et al. (2005)
Osorezan dace, *T. hakonensis*, FW, pH 3.5 water				↑ mRNA			Hirata et al. (2003)
Hagfish, *Myxine glutinosa*, metabolic acidosis					↑ mRNA		Edwards et al. (2001)
Salmon, *Salmo salar*, SW, respiratory acidosis						↑ mRNA	Seidelin et al. (2001)

Unless otherwise stated, changes apply to protein levels. FW, freshwater; SW, seawater; MRC, mitochondria rich cell; PVC, pavement cell; NC, no change.

apical membrane of PNA^+ MRCs) and an increase in functional NHEs or V-type H^+-ATPases (because of their presumed association with the apical membrane of PNA^- MRCs). Conversely, during alkalosis, an increase in the apical membrane surface area of PNA^+ MRCs exposed to the environment is postulated to occur together with a reduction in the apical membrane surface area of exposed PNA^- MRCs. The net consequence of such changes is suggested to be a decrease in net acid efflux owing to the combined effects of increased Cl^-/HCO_3^- exchange and a decrease in Na^+-linked H^+ extrusion (either via Na^+/H^+ exchange or H^+-ATPase coupled to a Na^+ channel). It is also possible that increased activity of basolateral V-type H^+-ATPase could contribute to reduced acid excretion (Tresguerres et al., 2005) at such times.

It is important to point out that, given the enormous diversity among fish species, a universal model of branchial acid–base regulation is unlikely. Indeed, distinct differences between elasmobranch and teleost fish have already been noted. However, although the model presented in Fig. 2 certainly will not apply to all species, it seems plausible that certain elements will emerge from this model that are common across species.

4. Renal acid–base regulation

Adjustments of renal acid secretion in water-breathing fish constitute an important complement to the metabolic compensation of acid–base disturbances that occurs at the gills. In particular, branchial acid excretion would become a futile exercise in the absence of a renal mechanism for the retention of accumulated HCO_3^- ions. The potential for renal mechanisms to contribute to acid–base regulation appears to be highest in freshwater fish, where large volumes of urine are produced as a strategy for coping with a hypo-osmotic

environment. In marine elasmobranchs and teleosts, the regulatory capacity of the kidney is limited by low urine flow rates and an apparent lack of responsiveness to systemic acid–base disturbances (Swenson, 2003). By contrast, the responses of the freshwater teleost kidney to acid–base disturbances are comparable in pattern and flexibility to those of the mammalian kidney (Wood et al., 1999), although quantitatively subservient to those of the gill (Claiborne et al., 2002). Whereas a metabolic alkalosis triggers net base excretion via the kidneys, enhanced renal acid excretion accompanies a systemic acidosis. Moreover, paralleling the differential responses of the mammalian kidney to respiratory versus metabolic acidoses, the rainbow trout kidney responded to a metabolic acidosis with enhanced acid excretion largely in the form of NH_4^+, but a respiratory acidosis with greater proton efflux coupled to inorganic phosphate excretion (Wood et al., 1999). The flexibility of these responses is underlined by the very different effects of metabolic alkalosis and respiratory acidosis on renal HCO_3^- reabsorption, despite similar plasma HCO_3^- levels in each case (Wood et al., 1999). Questions emerging from this work are focused on the regulatory mechanisms responsible for eliciting appropriate renal responses, with cortisol mobilization being of interest (Wood et al., 1999) (as at the gill; see above), as well as the molecular mechanisms through which urine acid–base status is manipulated.

A key component of the regulation of urine acid–base status is the reabsorption of filtered HCO_3^- ions. Recent work suggests that the mechanism of renal HCO_3^- reabsorption in rainbow trout in many ways parallels that of the mammalian proximal tubule, the site responsible for 80–90% of renal HCO_3^- reabsorption in mammals (Romero and Boron, 1999; Swenson, 2000). In particular, renal HCO_3^- reabsorption in rainbow trout relies on both cytosolic and membrane-bound CA isoforms (Georgalis et al., 2006a). Filtered HCO_3^- ions in the mammalian proximal tubule combine with secreted protons in a reaction catalyzed by type IV CA, a high activity enzyme linked to the plasma membrane by a GPI anchor (Schwartz, 2002). The resultant molecular CO_2 enters the renal tubule cell and is hydrated to H^+ and HCO_3^- in the presence of the high activity cytosolic type II CA isoform (Schwartz, 2002). While the protons are recycled into the lumen via an apically located Na^+/H^+ exchanger (NHE3) and/or V-type H^+-ATPase, HCO_3^- ions are moved across the basolateral membrane by a Na^+-HCO_3^- cotransporter (NBC1) resulting in net HCO_3^- transfer from lumen to blood (Romero and Boron, 1999; Swenson, 2000). Several elements of this model (Fig. 3) have now been identified in rainbow trout. Trout NBC1 has been cloned, and kidney tissue exhibits high levels of NBC1 mRNA expression (Perry et al., 2003a). The V-type H^+-ATPase has been localized to rainbow trout kidney tissue at both the mRNA and protein (using a heterologous antibody) levels (Perry and Fryer, 1997; Perry et al., 2000). Most recently, a high activity cytoplasmic CA isoform (tCAc; Esbaugh et al., 2005) as well as CA IV (tCA IV) were cloned from rainbow trout, and found to be expressed at both the mRNA and protein levels in proximal tubules (Georgalis et al., 2006a). Moreover, selective inhibition of tCA IV impaired urinary acid excretion, and even greater urinary loss of HCO_3^- occurred when cytoplasmic CA was also inhibited, implicating these proteins in the HCO_3^- reabsorption mechanism of the trout renal tubule (Georgalis et al., 2006a). Increased mRNA expression of each of these elements, NBC1, V-type H^+-ATPase, tCAc and tCA IV, as well as increased tCAc and tCA IV protein levels, occur during a respiratory acidosis, contributing to the necessary enhancement of HCO_3^- reabsorption (Perry et al., 2003a; Georgalis et al., 2006a). Although NHE3 has not yet been identified in rainbow trout, it was cloned from Osorezan dace and renal NHE3 mRNA expression was increased by exposure of dace to acidified water, suggesting that upregulation of this protein contributes to the response to a metabolic acidosis (Hirata et al., 2003). Thus, localization of NHE3 and NBC1 proteins to the apical and basolateral membranes, respectively, of renal tubule cells remains to complete the model of HCO_3^- reabsorption in fish kidney.

While the kidney plays a critical, but supporting, function in the response of water-breathing fish to acid–base disturbances, it might be expected to take on a more prominent role in air-breathing fish species, particularly those exhibiting reduced gills and greater dependence on air breathing (Graham, 1997; Brauner et al., 2004a). To date, however, few studies have attempted to test this hypothesis. Although Cameron and Wood (1978) examined the renal response to hypercapnia in the facultative air-breather *Hoplerythrinus unitaeniatus*, low animal numbers limited their ability to draw conclusions. Recently, renal and branchial

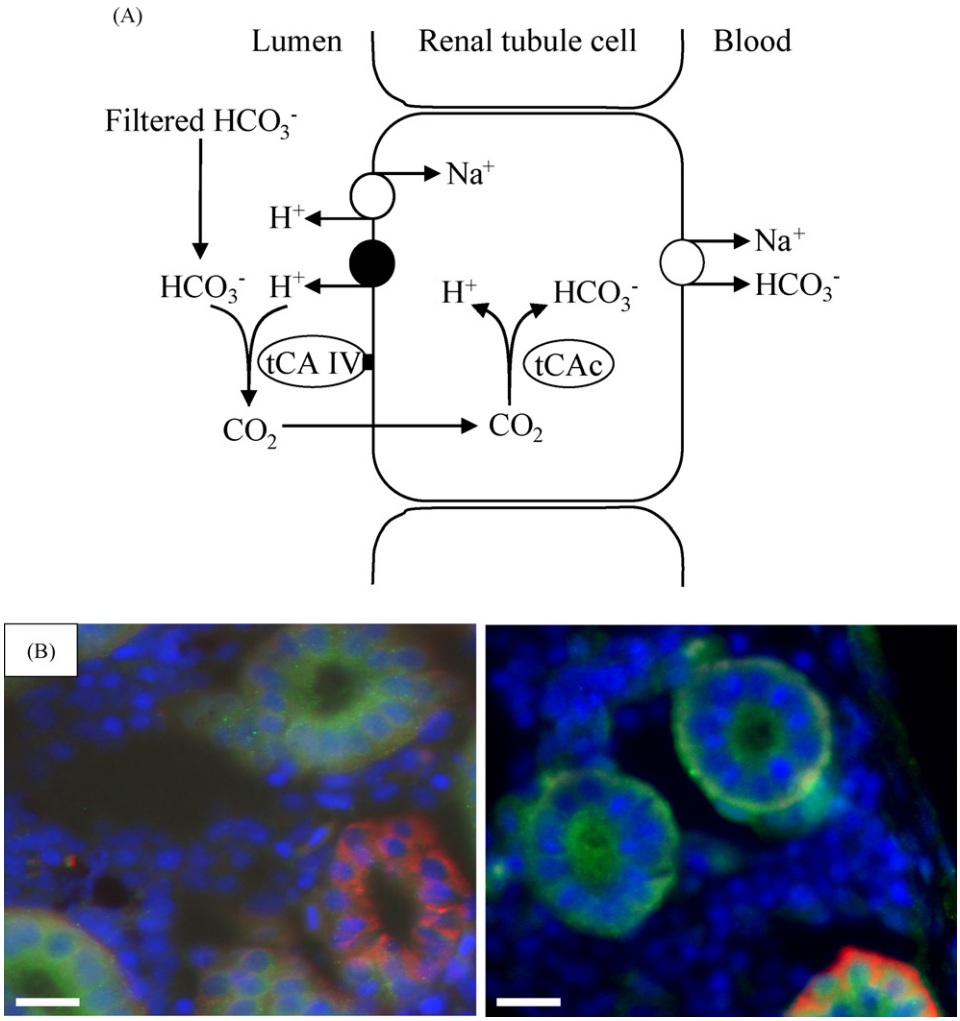

Fig. 3. (A) A model depicting the proposed mechanism of renal HCO_3^- reabsorption in fish. Acid added to the filtrate by the V-type H^+-ATPase and NHE3 titrate filtered HCO_3^- in the tubule lumen in a reaction catalyzed by membrane-bound CA IV. The resultant CO_2 enters the renal epithelial cell by diffusion and is hydrated to H^+ and HCO_3^- in the presence of a high activity cytoplasmic CA isoform (tCAc). Finally, HCO_3^- ions are moved across the basolateral membrane by NBC1, while protons are recycled into the tubule lumen. Evidence for all elements of this mechanism now exists for fish (see text), although NHE3 and NBC1 proteins must still be localized to the appropriate membranes. Energy-consuming transporters are indicated by filled circles. (B) Representative light micrographs revealing the presence of rainbow trout (*Oncorhynchus mykiss*) CA IV (tCA IV; left image) and cytoplasmic CA (tCAc; right image) in renal tubules by immunohistochemistry. The images depict CA immunoreactivity in green, immunoreactivity against α5, an antibody commonly used to detect Na^+, K^+-ATPase, in red, and nuclei, visualized using 4′,6′-diamidino-2-phenylindole, in blue. Areas of overlap of CA and α5 immunoreactivity are indicated by yellow/orange. Tubules varied in the extent to which either CA isoform was co-localized with α5. Note that the localization of CA activity within the cell cannot be inferred from these images—they simply indicate that two different isoforms of CA are present. Scale bar = 25 μm.

contributions to the correction of a metabolic alkalosis were examined in African lungfish, *P. annectens* (S.F. Perry, R. Euvermann, S.F. Chew, Y.K. Ip, K.M. Gilmour, unpublished data). Fish fitted with a urinary catheter and intra-arterial cannula were infused with $NaHCO_3$ over a 1 h period. Acid excretion into the water, which included contributions of the gills and skin, and urinary acid excretion were then assessed for a 4 h period immediately following base infusion, and for a 4 h period 17 h later. Lungfish responded to the

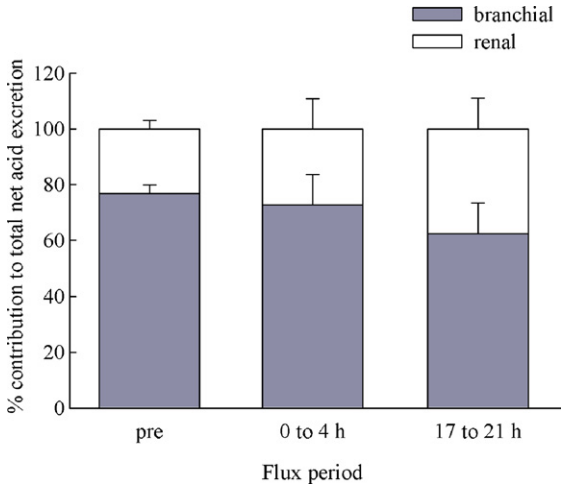

Fig. 4. The mean percentage contribution of gills/skin and kidneys in the African lungfish *Protopterus annectens* to the excretion of acid–base equivalents under control conditions ("pre") and 0–4, or 17–21 h, following the induction of a metabolic alkalosis by infusion of a base load. Net acid excretion into the water (by gills and skin) or urine was determined as the sum of net titratable acidity plus NH_4^+ excretion, signs considered. Values are mean ± 1 S.E.M., $N = 6$.

metabolic alkalosis by excreting base via both gills/skin and kidney. The branchial response was more rapid in onset than that of the kidneys, with net base excretion apparent in the 4 h period immediately following base infusion, a time during which the kidneys continued to excrete a small amount of acid. Interestingly, the gill and/or skin contribution (Fig. 4), while lower than the >90% quoted for unimodal water-breathers (Claiborne et al., 2002), was still the dominant route for the net excretion of acid–base equivalents both following an acid–base disturbance and under control conditions. These data may suggest that the gills of lungfish, while highly reduced, still play an important role in acid–base regulation, and given the tight linkages between the excretion of acid–base equivalents and ion uptake, presumably also ionic regulation. The potential contribution of the skin should not, however, be overlooked, as mitochondria-rich cells are abundant in both the skin and gills of *P. annectens* (Sturla et al., 2001).

5. Conclusions

Fish compensate for acid–base disturbances with a suite of integrated responses, the centrepiece of which is the direct transfer of acid–base equivalents across the gill. Through the manipulation of branchial transepithelial exchanges of acid–base equivalents for environmental NaCl via mechanisms that have not yet been fully described, fish adjust plasma HCO_3^- levels appropriately to correct imbalances in plasma pH. Complementing this mainstay of metabolic compensation is the renal response, which is particularly important in freshwater fish responding to an acidosis, where the upregulation of renal HCO_3^- reabsorption from the filtrate enables HCO_3^- ions accumulated at the gill to be retained. Owing to generally low Pa_{CO_2} values and an apparent absence of CO_2/pH-sensitive chemoreceptors oriented to monitor the blood, respiratory compensation appears to contribute little to the correction of acid–base disturbances in water-breathing fish. There are, however, a few studies that suggest otherwise and this area certainly warrants further investigation. In addition, respiratory compensation may play a larger role in air-breathing fish, likely owing to both the generally higher Pa_{CO_2} values exhibited by these fish and the apparent ability to uncouple O_2 uptake (via the air-breathing organ or lung) from CO_2 excretion (via the gills). The outstanding questions, however, with respect to pH balance and CO_2 excretion in fish, concern the molecular mechanisms through which acid–base equivalents are exchanged for environmental NaCl at the gill and the regulatory factors through which these processes are adjusted to achieve the necessary metabolic compensation of acid–base disturbances.

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