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Tribute to R. G. Boutilier: Acid–base transfer across fish gills

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Summary

The gills are the major site of acid–base regulation in most fish. Acid–base transfer across fish gills is dominated by carbon dioxide and ammonia excretion, especially the former. Bicarbonate buffering in the blood is less than that found in mammals; regulation of ventilation has little effect on CO_2 levels in the blood and control of ventilation is not used to regulate body pH in fish. Proton ATPase (freshwater fish), Na^+/H^+ exchangers (marine fish) and anion exchangers (marine and freshwater fish) are located in the gills. These transporters contribute to the regulation of internal pH, but little is known about how this is done in fish. Fish kept in confined water volumes acidify their environment, largely due to CO_2. This acidification augments ammonia excretion and reduces ammonia toxicity. The possible involvement of ammonia recycling in acid excretion is also discussed.

Key words: acid–base, gills, fish, ammonia, carbon dioxide.

Introduction

Bob Boutilier published extensively in the area of acid–base regulation and carbon dioxide movement in fish and amphibians, working in collaboration with many people including Dan Toews, Norbert Heisler, Graham Shelton and David Randall. This work began when Bob was an undergraduate and Shelton and Randall visited Dan Toews' laboratory in Acadia University for the summer of 1976 to investigate carbon dioxide excretion and plasma pH regulation in toads. Bob had just graduated at the time but we published four papers as a result of that work, one in *Respiration Physiology* and three in *The Journal of Experimental Biology*, with Bob as the lead author (Boutilier et al., 1978; Boutilier et al., 1979a; Boutilier et al., 1979b; Boutilier et al., 1979c). Bob had published eight papers before he completed his Masters degree at Acadia in 1979.

During Bob Boutilier's tenure as a Research Fellow at the University of British Columbia (1982–1984) we decided that we needed a better quantification of the physicochemical parameters associated with fish respiration. Bob put together a review (Boutilier et al., 1984) and we investigated the carbon dioxide hydration/dehydration reaction in fish plasma (Boutilier et al., 1985). These physical measurements formed a basis for many future studies. What became clear was that the pH of plasma in fish (and other vertebrates) was midway between the pK of the CO_2/HCO_3^- and NH_4^+/NH_3 reactions and that the transfer of acid and base across the gills was dominated by these same molecules (Randall and Wright, 1990). Ammonia excreted across the gills raises water pH, whereas carbon dioxide excretion lowers the pH of water as it passes over the gills. Carbon dioxide has the dominant effect because it is excreted in greater quantities than ammonia. More recently we have shown that if fish are placed in a limited water volume they will acidify the water, reducing pH to around 7.0 within a few hours. Carbon dioxide excretion and the associated acidification of gill water augments ammonia excretion (Fig. 1), due to ammonium ion trapping (Playle and Wood, 1989; Wright et al., 1989; Wilson et al., 1994).

Carbon dioxide can also be excreted in the form of bicarbonate through an anion exchanger on the gill apical membrane. This is generally considered to be small compared with the flux of carbon dioxide. Ammonia can be excreted as ammonia or ammonium ions. Once again excretion is generally dominated by the much more permeable unionized form, but it is possible that 2–3% of ammonia is excreted by ammonium ion diffusion in freshwater fish, with higher levels in seawater fish. In both carbon dioxide and ammonia excretion, transfer of the unionized form (CO_2 and NH_3) dominates and the usually small portion excreted in the ionized form (NH_4^+ and HCO_3^-) is both difficult to quantify and rarely estimated. In a few species of fish active NH_4^+ efflux dominates ammonia excretion (Randall et al., 1999).

Mudskippers acidify their burrows as a result of CO_2 and proton excretion. Fish may modulate acid excretion to regulate the pH of the water in the burrow, as suggested for the mudskipper *Periophthalmodon schlosseri* (Chew et al., 2006). To test this we placed individual fish in small volumes of water and measured water pH, CO_2 excretion and respiratory exchange ratios, using a variety of fish species.

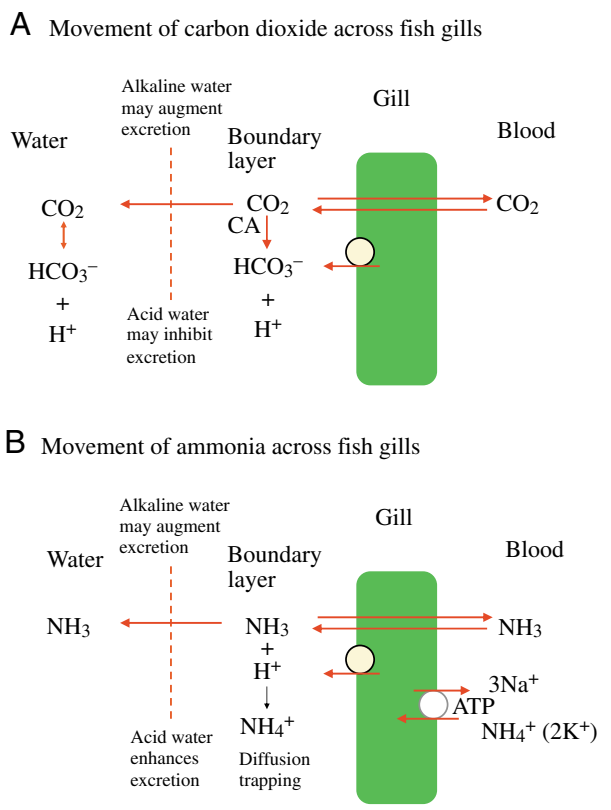


Fig. 1. Model of the movement of (A) carbon dioxide and (B) ammonia across fish gills. CA, carbonic anhydrase.

Materials and methods

Collection and maintenance of specimens

Oriental weatherloach *Misgurnus anguillicaudatus* Cantor and goldfish *Carassius auratus* L. were purchased locally from a wet market and were kept indoors (12 h:12 h light:dark) in dechlorinated tapwater at 20°C. They were fed with dry pellets (Hai Feng Quick Grow Gold Fish Food, Nantou, Taiwan) and one half of the water was changed daily, except for Sunday. Toadfish *Opsanus beta* Goode and Bean were purchased from a fish import company in Singapore and were kept indoors (12 h:12 h light:dark) in 30% seawater at 25°C. They were fed with live guppies and one half of the water was changed once every 2–3 days. Mudskippers, *Periophthalmodon schlosseri* Pallas and *Boleophthalmus boddaerti* Pallas were purchased from fishermen on the Malaysian Peninsular and kept indoors (12 h:12 h light:dark) in 30% seawater at 25°C. They were fed with live guppies and one half of the water was changed once every 2–3 days.

Measurement of environmental acidification brought about by fish

Individual fish were placed in a container of suitable size and shape so that the fish was fully submerged in a volume of water that was equivalent to 10 times the weight of the fish. Water pH was measured at specific time points using a pH electrode (model: Orion 91-05, Waltham, MA, USA) connected to a pH meter (model: Orion 250Aplus). Aeration was not provided during the experiment. Two containers containing approximately equal volumes of water but without fish were set up to serve as controls. Water used for freshwater species (weatherloach and goldfish) was dechlorinated tapwater with 1 mmol l^{-1} Hepes, adjusted to pH 7.7 with NaOH, the buffer increasing the stability of the pH measurement. For brackish water species (mudskippers and toadfish), aerated 50% seawater was used. To find out if the acidification was due to carbon dioxide excretion, proton excretion or both, the fish was removed from the container at the end of the pH monitoring period. An air stone with vigorous air supply was placed in the individual container in order to remove any carbon dioxide produced by the fish. Water pH was again measured after 1 h of bubbling to compare with the control water.

The effects of pH and ammonia on the respiratory exchange ratio

A weatherloach was placed in a water-filled enclosed respiratory chamber without any air bubbling. A small magnetic bar was placed in the chamber to facilitate mixing within the chamber. Oxygen content at the beginning and the end of the experiment was measured using a Clark-type oxygen electrode (YSI, Yellow Springs, OH, USA). Carbon dioxide content was measured using a gas chromatograph method. Briefly, a water sample was mixed with 1 mmol l^{-1} HCl in an enclosed syringe and the syringe water was shaken vigorously for about 10 s so that all carbon dioxide was driven into the gas phase in the syringe. The gas in the syringe was then removed and immediately injected into a gas chromatograph. Sodium bicarbonate was used as a standard. All water samples were analyzed immediately after collection, as a preliminary experiment showed that delay in analysis resulted in loss of carbon dioxide. The respiratory exchange (RE) ratio was obtained by dividing carbon dioxide excretion rate by oxygen consumption rate.

For measurement of the effect of water pH on RE, water containing 2 mmol l^{-1} Tris, pH adjusted to 7.0, 8.0 or 9.0 with HCl, was used. For measurement of the effect of ammonia on RE, water containing 10 mmol l^{-1} Tris and 15 mmol l^{-1} ammonium chloride, pH adjusted to 7.0 with HCl, was used. The time course of each experiment was 30–40 min. The fish:water volume ratio was 1:60. The pH of the water was checked at the beginning and the end of experiments and found to have changed by less than 0.1 pH unit.

Statistical analysis

Results are presented as means ± s.e.m. Student's *t*-test or one-way analysis of variance (ANOVA) followed by Student–Neuman–Keul's multiple range test were used to compare differences between means where applicable. Differences with $P<0.05$ were regarded as statistically significant.

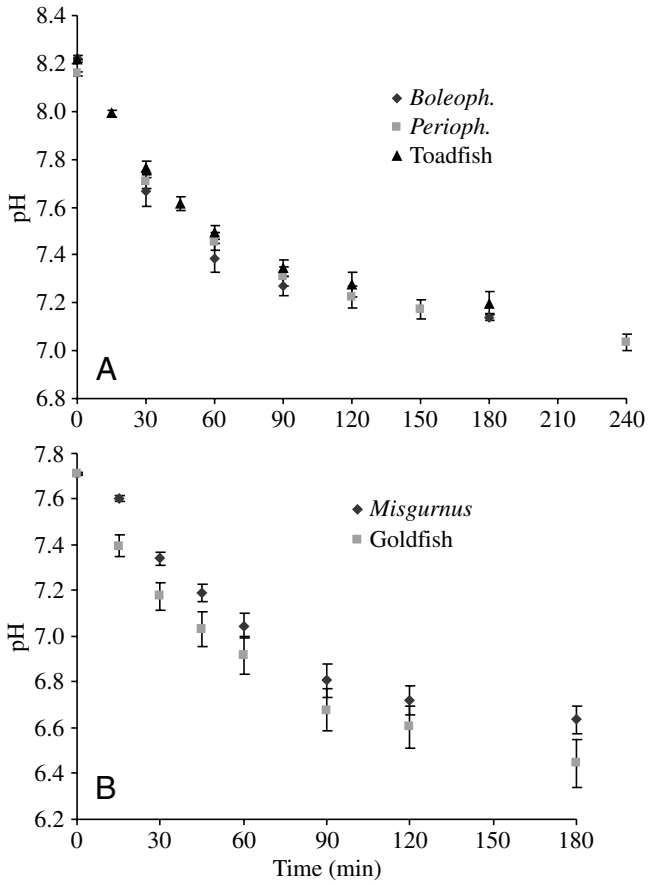


Fig. 2. Acidification of the ambient water by different fish species. (A) Brackish water species. (B) Freshwater species. Boleoph., *Boleophthalmus boddaerti*; Perioph., *Periophthalmodon schlosseri*; Toadfish, *Opsanus beta*; Misgurnus, *Misgurnus anguillicaudatus*; Goldfish, *Carassius auratus*. Values are means ± s.e.m., *N*=6. All data points after 15 min are significantly different from those at time 0.

Results and discussion

It was found that pH of the ambient water decreased gradually over time (Fig. 2). The same phenomenon was observed for both freshwater fish and brackish water fish. The rate of pH decrease depended on the water volume in relation to the size and activity of the fish. The rate and magnitude of pH change also depends on the buffering capacity of water, the ratio of CO_2 to NH_3 excreted, and the water flow rate and mixing as it passes over the gills. Aeration of the water after exposure returned the pH to the original value (data not shown), indicating that the acidification was caused by carbon dioxide excretion, as expected, and that any contribution from proton excretion was negligible. There was no significant effect of water pH on oxygen uptake, carbon dioxide excretion or respiratory exchange ratio in the oriental weather loach *Misgurnus anguillicaudatus* (Table 1). Thus the pH changes in the water appear to be simply the physico-chemical consequence of carbon dioxide excretion.

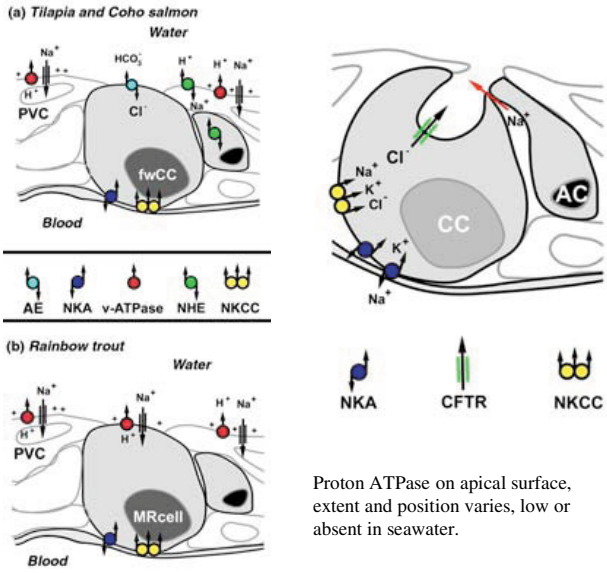
Fish living in enclosed spaces, such as the mudskipper *Periophthalmodon schlosseri*, live in acidified conditions. The extent of acidification is also influenced by burrow position and design, which determines flushing rates of the burrow. Water from the *P. schlosseri* burrow was reported to be about pH 7.0 and the ammonia concentration approximately 3 mmol l^{-1} (Ip et al., 2004). The water was hypercapnic, indicating that at least part of the acidification process was due to CO_2 excretion. Net acid production by *P. schlosseri* in laboratory experiments was reduced by addition of the V-ATPase blocker, bafilomycin, indicating that proton excretion was also contributing to the acidification process. Raising water pH also increased net acid production by the fish and this was partially blocked by bafilomycin, indicating that there was an increase in proton excretion with increasing water pH. This was also observed in trout; proton excretion across the gills increases with water pH (Lin and Randall, 1995). A portion of the increase in acid excretion associated with elevated water pH was bafilomycin insensitive and, in addition, increases in ammonia concentration in water to 20 and 30 mmol l^{-1} resulted in a large increase in acid excretion. A possible explanation is that active NH_4^+ excretion is coupled to a backflux of NH_3 into the animal, this ammonia recycling resulting in a net acid excretion but no change in ammonia excretion.

Table 1. *Effects of pH and ammonia on oxygen consumption, carbon dioxide excretion and respiratory exchange ratio (RE) of Misgurnus anguillicaudatus*

Conditions	O_2 consumption	CO_2 excretion	RE
pH 7.0	0.071±0.013	0.064±0.001	1.05±0.23
pH 8.0	0.064±0.005	0.059±0.008	0.92±0.10
pH 9.0	0.076±0.009	0.075±0.011	0.93±0.06
pH 7.0 + 15 mmol l^{-1} NH_4Cl	0.081±0.008	0.085±0.008	1.05±0.05

Units of O_2 consumption and CO_2 excretion are μmol min^{-1} g^{-1} fish.
Values are means ± s.e.m., *N*=6.

Active ammonium ion excretion by the mudskipper *P. schlosseri* occurs in the head region, probably across the gills (Ip et al., 2004). The gills contain very high levels of Na^+-K^+-ATPase (NKA) in the basal–lateral membrane, which can be activated by ammonium, and high levels of $Na^+/H^+(NH_4^+)$ exchanger (NHE) in the apical membrane (Randall et al., 1999). Ammonium excretion can be blocked by ouabain and amiloride. It is suggested that ammonium ions are removed from the blood *via* a Na^+-$K^+/(NH_4^+)$-ATPase and then cross the apical membrane *via* an NHE. Ammonia recycling could account for both the bafilomycin insensitive and the ammonia stimulated increases in acid excretion across the gills. In ammonia recycling there would be a backflux of NH_3 in the face of active NH_4^+ excretion, resulting in a net acid excretion but reduced ammonium excretion across the gills. Water NH_3 levels increase with pH and total ammonia concentration, increasing NH_3 backflux and, therefore, acid excretion. Thus reductions in water pH in the mudskipper burrow are probably related to CO_2 and proton excretion and ammonia recycling.



NHE apical and widely distributed

Proton ATPase on apical surface, extent and position varies, low or absent in seawater.

Fig. 3. Location of various transporters on the gills of tilapia (A, left) coho salmon (A, right) and rainbow trout (B). CFTR, cystic fibrosis transmembrane regulator-like anion channel; NKA, Na^+/K^+ ATPase; AE, anion exchanger; V-ATPase, a proton pump: NHE, a Na^+/H^+-like exchange carrier; NKCC, an $Na^+/K^+/2Cl^-$ cotransporter; PVC, pavement cell; fwCC, freshwater chloride cell; CC, chloride cell; MR cell, mitochondria-rich cell; AC, accessory cell. (Modified from Wilson and Laurent, 2002.)

The contribution to acid excretion of proton and CO_2 excretion and ammonia recycling will diminish with reductions in pH in water, maintaining the burrow pH at around 7.0.

Active ammonium ion excretion could be expensive if there was a large backflux of ammonia into the fish. Backflux of ammonia can be reduced by (i) acidification of the environment through CO_2 and proton flux and ammonia recycling into a limited water volume, to trap ammonium ions, and (ii) a reduction in skin permeability to gases. *P. schlosseri* has a low skin NH_3 permeability (Ip et al., 2004), at least in the tail region. This air breathing fish can survive in burrows containing hypoxic water with high ammonia levels that would kill other animals (Randall et al., 2004).

Acid excretion across the gills

In addition to the acid–base effects of CO_2 and NH_3, carnivorous fish normally have a net acid excretion whereas herbivorous fish have a net base excretion. Much of this acid–base regulation in fish occurs across the gills, rather than *via* the kidney as in terrestrial vertebrates. Excretion across the gills is facilitated by a large water flow, which is several orders of magnitude larger than urine flow, even in freshwater fish. In fact about 90% of compensatory net transfers occur across the gills and only 10% *via* other pathways, including the kidney (Evans, 2005). Fish have low levels of bicarbonate buffering compared with mammals, but an acidosis is accompanied by plasma bicarbonate dehydration and an associated increased carbon dioxide excretion. Blood carbon dioxide levels are normally low and there is little effect of changing ventilation on carbon dioxide levels, thus ventilation plays little role in acid–base adjustments in fish, as demonstrated by Bob Boutilier and others working on the trout (Iwama et al., 1987). There is, however, extensive use of acid–base transfer across gills to regulate plasma pH. There is a low net flux of non-volatile acid normally and a capacity to increase acid or base flux by an order of magnitude, given an acid or base load (Evans, 2005).

Proton excretion across the gills is pH dependent; alkaline conditions in the environment and increased cell acidity promote increased proton excretion (Lin and Randall, 1995). Proton excretion is inhibited in trout at pH 5.5 in water (Lin and Randall, 1991), but many fish live in lower pH waters (Gonzalez et al., 1998; Kaneko et al., 1999; Yada and Ito, 1999). There is a large flux of carbonate into the gut of fish exposed to high intestinal Ca^{2+} levels, resulting in the formation of insoluble $CaCO_3$, which is excreted with the feces, ameliorating Ca^{2+} influx into the fish across the gut wall (Wilson and Grosell, 2003). The acid–base status of the fish, however, is not affected by this large carbonate loss as it is associated with an equivalent flux of acid across the gills (Wilson et al., 1996).

The gills of many freshwater fish have been shown to contain V-type proton-ATPase on their apical surface (Figs 3, 4) with co-localization of carbonic anhydrase (Lin et al., 1994). The actual distribution varies both in terms of cell type and location on gills between fish (Wilson and Laurent, 2002). Changes in acid excretion are associated with changes in the area of exposed gill surface membrane (Goss et al., 1995). Proton excretion is believed to increase *via* exocytotic insertion of proton ATPase into the surface membrane, similar to that reported in turtle bladder (Cannon et al., 1985) and frog skin (Harvey, 1992). Cortisol has been shown to be associated with increased proton ATPase activity in trout gills (Lin and Randall,

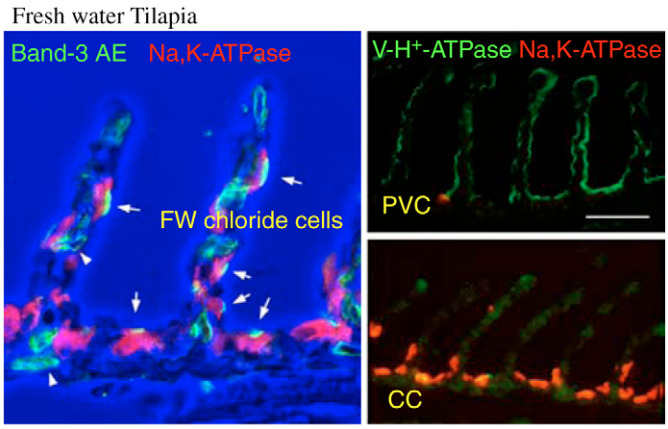


Fig. 4. Immunolocalization of various transporters in freshwater tilapia gill. CC, chloride cell; PVC, pavement cell. Band-3 AE is an anion exchanger; V-H^+-ATPase is a proton pump (Wilson et al., 2000). Arrowheads indicate freshwater chloride cells.

1993). There is also increased expression of proton ATPase following an acid load (Perry et al., 2003a) and sometimes following hypercapnia (Sullivan et al., 1996; Perry et al., 2000; Galvez et al., 2002). Proton ATPase activity decreased after transfer of salmonids to seawater (Wilson et al., 2002).

In seawater fish, with reduced or absent proton ATPase activity, Na^+/H^+ exhangers (NHEs) in the gill apical membrane are involved in proton transfer and show an increased expression with acid load (Edwards et al., 2001). Some fish have been reported to have a Na^+/HCO_3^- cotransporter (NBC1) in the gill basal membrane (Perry et al., 2003b). It is possible that something similar to the mammalian B-type cell has been observed in killifish with V-type ATPase on the basal-lateral membrane for base excretion (Katoh et al., 2003), but this has not been found in the majority of fish studied. The gill of Atlantic stingray *Dasyatis sabina* has been shown to have an H^+-K^+-ATPase, which is thought to aid acid excretion (Choe et al., 2004). It is clear that there are at least two types of chloride cell in teleost fish (Galvez et al., 2002) and elasmobranches (Piermarini and Evans, 2001).

The Japanese osorezan dace can live in acid water of pH 3.5 (Kaneko et al., 1999). There are NHE3 transporters in the apical membrane of the gills associated with carbonic anhydrase (CA), and NBC1 as well as NKA are found in the basolateral membrane (Hirata et al., 2003). There appears to be increased local synthesis of ammonia when the fish is in acidic water as mRNA of glutamate dehydrogenase (GDH), an enzyme involved in glutamine degradation and ammoniagenesis, was increased in all tissues examined (Hirata et al., 2003). It is possible that alkalinization of boundary layer by ammonia or ammonium ion excretion *via* NHE will augment acid excretion in this fish and enable it to survive in very acid conditions.

Even less is known about base excretion in fish. Base excretion has been shown to increase with base load *via* anion exchangers (AE) with chloride as the counter ion. There is increased expression of AE in the gills of rainbow trout following a base load (Sullivan et al., 1996). Some fish excrete carbonate *via* the gut (Wilson and Grosell, 2003), but this is considered to be related to calcium homeostasis rather than acid–base regulation.

It is clear that the gills are the major site of acid–base regulation in fish. In addition, several mechanisms, such as the V-type ATPase, have been described. To understand how these mechanisms are integrated to achieve acid–base homeostasis, however, will require much more work.

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