

**EFFECT OF COPPER ON CYTOCHROME P450 1A-CATALYSED
XENOBIOTIC BIOTRANSFORMATION AND BARRIER
PROPERTIES IN RAINBOW TROUT GILL CELLS**

Maria Jönsson
Uppsala University, Evolutionary Biology Centre,
Department of Environmental Toxicology, Norbyvägen 18A,
S-752 36 Uppsala, Sweden.
Tel: +46-(0)18-471 26 18 Fax: +46-(0)18-51 88 43
E-mail: Maria.Jonsson@EBC.uu.se

Carina Carlsson¹, Richard W. Smith^{2†} and Peter Pärt³
† = current address

1 Environmental Toxicology, Norbyvägen 18A,
S-752 36 Uppsala, Sweden.

2 University of Aberdeen, Department of Zoology,
Tillydrone Avenue, Aberdeen, Scotland, UK.

3 † European Commission, Joint Research Centre (C.C.R.),
Environment Institute, Ispra, Italy

EXTENDED ABSTRACT ONLY - DO NOT CITE

The gill is a site for uptake and toxic action of waterborne contaminants, including metals and organic pollutants. Metals, such as silver, cadmium and copper, disrupt ion regulation and cause histological damage, whilst dioxins, polychlorinated biphenyls (PCBs) and polychlorinated aromatic hydrocarbons (PAHs) induce cytochrome P4501A (CYP1A). Polluted bodies of water often contain a mixture of different chemicals, many of which are mutually interactive. For example, metals can reduce the CYP1A-activity, as is the case with copper in bass (*Dicentrarchus labrax*) liver and hepatocyte microsomes (Stien et al., 1997; Viarengo et al., 1997).

In this study, copper effects on the CYP1A-catalysed reaction, 7-ethoxyresorufin *O*-deethylation, (EROD), were examined in rainbow trout (*Onchorhynchus mykiss*) gills *in vitro*. Gill cells were cultured as polarised tight

epithelia on permeable supports; *i.e.* with apical and basolateral compartments (Wood and Pärt, 1997). These cultures, which 'tolerate' water at the apical membrane (Wood and Pärt, 1997), were exposed (24 hours) to aqueous copper and β -naphthoflavone (BNF; to induce CYP1A), via the apical the basolateral compartments, respectively. This cell model was also compared with conventional gill cell monolayers, grown in 12-well tissue culture plates (*i.e.* contacting culture medium with only one side). Monolayers were exposed for 24 hours to copper and BNF in the medium.

EROD-activity was determined (in intact adherent cells) according to Carlsson et al. (1999); *i.e.* water and media were replaced with 1 μ M 7-ethoxyresorufin and, after 20 minutes, resorufin concentrations were determined by fluorescence (at 544 nm ex and 590 nm em). In polarised gill epithelia resorufin is selectively distributed to the basolateral compartment (Carlsson and Pärt, submitted), and therefore apical and basolateral resorufin concentrations were measured separately. EROD-activity was calculated from the total resorufin concentration.

In polarised epithelia, the effects of copper on barrier properties (conductivity, Na^+ -efflux and polyethylene glycol (PEG)-permeability) were also investigated. Conductivity was obtained from the inverted transepithelial electrical resistance, measured with an epithelial voltohmmeter, connected to an Ag^+/AgCl 'chopstick' electrode. Na^+ -efflux is both transcellular and paracellular, whereas PEG-permeability is a paracellular marker (used to evaluate tight junction integrity). Na^+ -efflux and PEG-permeability were measured after addition of $^{22}\text{Na}^+$ and ^3H -PEG-4000 to the basolateral medium.

The exposure groups (within a culture type) and apical / basolateral resorufin distribution (in polarised epithelia) were statistically compared using two-tailed and one-tailed Wilcoxon matched pairs tests, respectively. Significant differences are indicated in the tables by superscript letters ($p < 0.05$) and stars (* = $p < 0.05$ and ** = $p < 0.01$ and *** $p < 0.001$).

EROD-activity in monolayers was reduced by exposure to 5–1000 μ M copper, whilst EROD-activity in polarised epithelia was unaffected by copper exposure (0.5–100 μ M; Table 1). However, the selective basolateral resorufin distribution levelled out following exposure to 75–100 μ M copper (Table 1).

Table 1. Copper effects on EROD-activity in rainbow trout gill cell cultures

Copper μM	Pmoles resorufin $\text{mg protein}^{-1} \text{min}^{-1}$					
	Cultured epithelia				Monolayers	
	EROD-activity ¹		Basolateral-apical difference ²		EROD-activity ¹	
	Median	Range	Median	Range	Median	Range
0	27 ^A	9–43	5*	(-11)–23	49 ^a	15–77
0.5	23 ^A	14–43	8*	(-1)–13		
1	17 ^A	9–41	7**	0–12	49 ^a	12–77
5	18 ^A	8–49	6**	(-1)–12	41 ^b	3–62
10	28 ^A	9–46	8**	(-6)–18	35 ^b	6–59
25	33 ^A	9–53	7*	(-9)–22		
50	31 ^A	10–53	7*	(-12)–12	35 ^{cd}	4–53
75	28 ^A	15–50	2	(-18)–15		
100	18 ^A	10–41	3	(-11)–12	40 ^{bc}	6–57
500					30 ^d	4–48
1000					7 ^e	2–35
0 (-BNF)	1 ^B	0–3	0	0–3	8 ^e	1–26

¹ Significant differences are indicated by superscript letters, ² or by stars.

PEG-permeability and Na^+ -efflux increased following exposure to 75 μM copper (to 140 and 127 % of control, respectively), whilst conductivity was unaffected by 25 and 75 μM copper (Table 2).

In monolayers EROD-activity was considerably more sensitive to copper than in polarised epithelia, but the reason for this is unknown. The low susceptibility of polarised epithelia, suggests that their capability to metabolise organic pollutants is retained during exposure to copper. However, the results indicate that changes of the barrier properties have occurred. We interpret the break-down of the apical / basolateral resorufin distribution pattern as being due to increases of epithelial permeability. This was supported by that Na^+ -efflux increased following exposure to 75 μM copper. The effect on PEG-permeability indicates more specifically that the tight junctions were targeted.

Table 2. Copper effects on conductivity, PEG-permeability and Na⁺-efflux in polarised gill epithelia.

Effect parameter	N	copper μM	Relative values % of control	
			Median ¹	Range
Na ⁺ -efflux	14	0	100	
		25	103	35–162
		75	140*	75–269
PEG-permeability	10	0	100	
		25	113	68–255
		75	127***	105–340
Conductivity	11	0	100	
		25	100	<43 ² –235
		75	122	43–299

¹ Significant differences are indicated by stars. ² Beyond the epithelial volt-ohm-meter limit.

The gill has been suggested to function as a first-pass defence to organic pollutants (dioxins, PCBs and PAHs), by reducing their bioavailability (*i.e.* lipophilicity) before they enter the systemic circulation (Levine and Oris, 1999; Carlsson et al., 1999). The present findings also support the idea that since the outermost branchial cell layer is intimately exposed to any hazardous physico-chemical variations in the surrounding water, these cells would need to have a high resistance to toxic action. Injury of the respiratory cells would reduce their plasticity and impair their ability to maintain barrier integrity and thus be an acute danger for the animal.

In conclusion, the results of this study suggest that both the CYP1A enzyme activity and the barrier properties of intact polarised gill epithelia have a low susceptibility to aqueous copper exposure. This could have implications for the tolerance of sub-lethal waterborne pollutants *in vivo*.

References

Carlsson, C., Pärt, P. and Brunström, B. 1999. EROD induction in cultured gill epithelial cells from rainbow trout *Aquatic Toxicology* 47, 117–128.

- Carlsson, C. and Pärt, P. 2000. 7-Ethoxyresorufin O-deethylase induction in rainbow gill epithelium cultured on permeable supports: Asymmetrical distribution of substrate metabolites. *Aquatic Toxicology*, submitted
- Levine, S.L. and Oris, J.T. 1999. CYP1A expression in liver and gill of rainbow trout, following waterborne exposure: implications for biomarker determination. *Aquatic Toxicology* 46: 279–287
- Stien, X., Risso, C., Gnassia-Barelli, M., Romeo, M. and Lafaurie, M. (1997) Effect of copper chloride *in vitro* and *in vivo* on the hepatic EROD-activity in the fish *Dicentrarchus labrax*. *Environmental Toxicology and Chemistry* 16: 214–219
- Viarengo, A., Bettella, E., Fabbri, R., Burlando, B. and Lafaurie, M. 1997. Heavy metal inhibition of EROD-activity in liver microsomes from the bass *Dicentrarchus labrax* exposed to organic xenobiotics; role of GSH in the reduction of heavy metal effects. *Marine Environmental Research* 44: 1–11
- Wood, C.M. and Pärt, P. 1997. Cultured branchial epithelia from freshwater fish gills. *Journal of Experimental Biology* 200: 1047–1059

**OSMOTIC AND IONIC REGULATION IN JUVENILE FISH
EXPOSED TO CYANOBACTERIAL TOXINS**

F B Eddy
Department of Biological Sciences
University of Dundee, Dundee, DD1 4HN, Scotland, UK.
f.b.eddy@dundee.ac.uk

J H Best and G A Codd
Department of Biological Sciences
University of Dundee, Dundee, DD1 4HN, Scotland, UK

EXTENDED ABSTRACT ONLY – DO NOT CITE

Juvenile salmonids inhabit a hypoosmotic environment and continuously lose salts by diffusion and gain water by osmosis. Even in recently hatched yolk sac fry (or alevins), achievement of hydromineral balance is by absorption of inorganic ions from the medium, possibly via mitochondrion rich cells on the body surface while excess water is excreted via the kidney. Alevins and fry drink freshwater, and although drinking has no obvious physiological function, it represents a route for water uptake, as well as for uptake of substances from the environment. Toxic substances in the water may enter directly via the body surface if they are sufficiently lipid-soluble, e.g. organic substances and unionised ammonia. Some toxins may inhibit ionic absorption processes in ion absorbing cells on the body surface, e.g. zinc, cadmium and the ammonium ion. Drinking represents another means of toxin presentation, with absorption via the gut mucosa, though little is known of this route, or its significance.

Cyanobacteria (blue green algae) produce a variety of toxins in fresh water, including neurotoxins and endotoxins and one group of cyclic peptides, the microcystins which are hepatotoxins, has been extensively studied in mammals. Following ingestion, symptoms include vomiting and diarrhoea followed by damage to liver parenchyma cells, which may be fatal. There have been fewer studies in fish and long term-exposure of brown trout to microcystin resulted in reduced growth rate and liver damage (Bury *et al* 1995). Microcystins are not sufficiently lipid soluble to enter via the body surface, but may enter via drinking and transport mechanisms in the gut (Bury *et al* 1997). Fish-kills

sometimes occur during blooms of cyanobacteria though it is difficult to attribute mortality to a single cause. Mortality could result from deterioration of water quality often characterised by lowered oxygen levels, increased ammonia and pH levels, and toxins which may be released during senescence of the bloom.

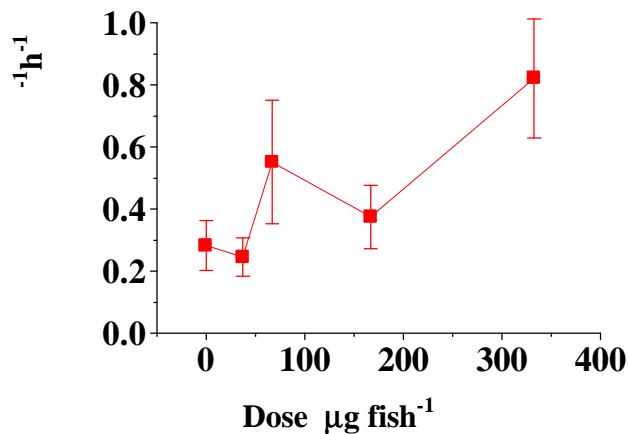


Fig 1. Effects of *Escherichia coli* LPS on drinking rate in juvenile rainbow trout

A variety of other substances are present during blooms and there is recent interest in lipopolysaccharides (LPS) produced by the cyanobacteria and bacteria present in blooms. Our studies aimed to investigate the effects of LPS on the osmoregulatory physiology of juvenile salmonids, with particular regard to the role of drinking. Exposure of juvenile rainbow trout to LPS of *E.coli* origin by injection, up to 300 μg per fish, resulted in significantly increased drinking although the effects of crude extracts of *Anabaena* bloom produced a less marked effect. It is considered that LPS may contribute significantly to fish kills. They may enter via the body surface on account of their lipid-solubility and then, possibly via a cytokine system, stimulate production of nitric oxide (NO) through stimulation of inducible nitric oxide synthase (iNOS). Increased levels of NO result in vasodilation, with subsequent stimulation of the renin-angiotensin system (RAS) and production of angiotensin II (AII), which stimulated drinking in both fresh water and marine fish (Fuentes and Eddy 1997). Thus cyanobacterial blooms may increase the drinking rate of fish so

allowing increased uptake of toxin via the gut which could be a contributory factor in mortalities. Physiological and environmental aspects of these events are explored.

References

- Bury, N.R., F.B. Eddy and G.A. Codd 1995. The effects of the cyanobacterium *Microcystis aeruginosa* and the cyanobacterial toxin microcystin-LR and ammonia on growth rate and ionic regulation of brown trout *Journal of Fish Biology*, 46:1042-1054.
- Bury, N.R., J.C. McGeer, F.B. Eddy and G.A. Codd 1997. Liver damage in brown trout *Salmo trutta* L., and rainbow trout *Oncorhynchus mykiss* (Walbaum), following administration of the cyanobacterial hepatotoxin microcystin-LR via the dorsal aorta. *Journal of Fish Diseases* 20: 209-215.
- Fuentes, J. and F.B. Eddy 1997. Effect of manipulation of the renin-angiotensin system in control of drinking in juvenile Atlantic salmon (*Salmo salar* L) in fresh water and after transfer to sea water *Journal of Comparative Physiology*. 167: 438-443.

**RAPID ACTIVATION OF GILL Na⁺,K⁺-ATPase
IN THE EURYHALINE TELEOST *Fundulus heteroclitus***

Juan Miguel Mancera
Departamento de Biología Animal,
Facultad de Ciencias del Mar, Universidad de Cádiz,
11510 Puerto Real, Cádiz, Spain,
E-mail: juanmiguel.mancera@uca.es

Stephen D. McCormick
Conte Anadromous Fish Research Center,
Biological Resources Division, USGS,
P.O. Box 796, Turners Falls, MA 01376, USA, E-mail:
mccormick@umext.umass.edu

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Fundulus heteroclitus is an estuarine euryhaline fish with the capacity to live in a wide range of environmental salinities. In its intertidal habitat the fish is subjected to large and often rapid changes in environmental salinity. Acclimation to changing environmental salinity requires pre-existing mechanisms and the ability to respond to changing conditions. Activation of gill chloride cells and regulation of gill Na⁺,K⁺-ATPase are very important for the acclimation of fish from fresh water to seawater (Wood and Marshall, 1994). In *Fundulus heteroclitus* an increase in gill Na⁺,K⁺-ATPase activity occurs 2-3 days after transfer from hypoosmotic to hyperosmotic conditions (Jacob and Taylor, 1983). In addition, a more rapid activation of gill Na⁺,K⁺-ATPase have been reported (Towle et al., 1977). However, in the same species Marshall et al. ('99) did not observe any change in this activity following exposure to seawater. The physiological significance and cellular mechanism for the rapid increase in Na⁺,K⁺-ATPase activity observed in the gill chloride cell is not clear. The aim of this study was to analyze the potential mechanisms of rapid activation of gill Na⁺,K⁺-ATPase in the euryhaline teleost *Fundulus heteroclitus*.

Materials and methods

Fundulus heteroclitus were transferred from low salinity water (LSW, 0.1 ppt salinity) to SW (35 ppt) and sampled for gill biopsy at 0 h (LSW-acclimated fish), 3 h, 6 h, 12 h, 24 h, 3 d and 7 d after transfer. In a second experiment fish were transferred and sampled at 1 h, 2 h, 3 h and 6 h. The rapid activation *in vitro* was examined using different osmolalities (300, 500, 600 and 800 mosm/kg) in gill organ culture. Samples were taken at 0 h, 3 h and 6 h of culture. In addition, the influence of several inhibitors (actinomycin D, cycloheximide and bumetanide at doses of 10^{-4} , 10^{-5} and 10^{-6} M) was analyzed in gill culture in hyperosmotic medium (600 mosm/kg) to determine the mechanisms involved in rapid activation of gill Na^+, K^+ -ATPase. Na^+, K^+ -ATPase activities were determined using the microassay method of McCormick (1993). The method of McCormick and Bern (1989) was used to culture primary gill filaments. Significant differences among groups were tested by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Two-way ANOVA and Student-Newman-Keuls multiple comparison test were used to test the significance of time and detergent treatment. Results were considered significantly different when $p < 0.05$.

Results

Exposure of *Fundulus heteroclitus* to SW induced a rise in gill Na^+, K^+ -ATPase activity 3 h after transfer. After 12 h the values dropped to initial levels but showed a second significant increase 3 d after transfer. The absence of detergent in the enzyme assay resulted in lower values of gill Na^+, K^+ -ATPase and the rapid increase after transfer to SW was not observed (Figure 1). Na^+, K^+ -ATPase activity of gill filaments *in vitro* for 3 h increased proportionally to the osmolality of the culture medium (600 mosm/kg > 500 mosm/kg > 300 mosm/kg). Osmolality of 800 mosm/kg resulted in lower gill Na^+, K^+ -ATPase activity relative to 600 mosm/kg. Increasing medium osmolality to 600 mosm/kg with mannitol also increased gill Na^+, K^+ -ATPase. Cycloheximide inhibited the increase in gill Na^+, K^+ -ATPase activity observed in hyperosmotic medium in a dose-dependent manner (10^{-4} M > 10^{-5} M > 10^{-6} M). Actinomycin D or bumetanide in the culture (doses of 10^{-4} , 10^{-5} and 10^{-6} M) did not affect gill Na^+, K^+ -ATPase (Figure 2). Injection of fish with actinomycin D prior to gill organ culture, however, prevented the increase in gill Na^+, K^+ -ATPase activity in hyperosmotic media.

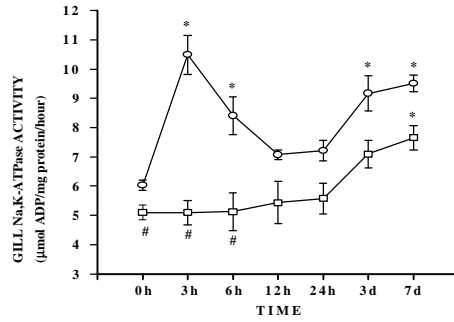


Figure 1. Changes in gill Na^+, K^+ -ATPase activity after transfer of *Fundulus heteroclitus* from LSW (0 h) to SW (35 ppt salinity). Gill Na^+, K^+ -ATPase activity was measured with detergent (0.1 % deoxycholic acid; circle) and without detergent (square). Each point represents mean \pm standard error (n=6-7 fish). * indicates significant difference from time 0 ($p < 0.05$, two-way ANOVA test and Student-Newman-Keuls multiple comparison test). # indicates significant difference between groups at the same time ($p < 0.05$, t- test).

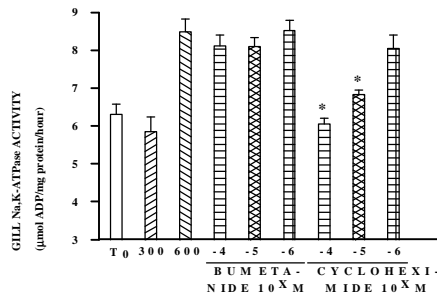


Figure 2. Effect of bumetanide and cycloheximide on gill Na^+, K^+ -ATPase activity after 3 h in culture using hyperosmotic medium (600 mosm/kg). Values are means \pm standard error (n=4-5). Asterisk indicates significant difference compared to hyperosmotic control medium ($p < 0.05$, one-way ANOVA test and Student-Newman-Keuls multiple comparison test).

Conclusions

The results of the present papers show a rapid and transitory increase in gill Na^+, K^+ -ATPase activity during first hours after transfer of *Fundulus heteroclitus* from LSW to SW. Similar results are obtained *in vitro* using a gill culture system after increasing medium osmolality. The origin of this increase in gill Na^+, K^+ -ATPase activity could involve modifications of pump catalytic subunits, changes in the subcellular distribution of pump units or increase in translational or post-translational kinetics. The results obtained with actinomycin D and cycloheximide suggests that the rapid activation of gill Na^+, K^+ -ATPase activity in *Fundulus heteroclitus* is dependent on transcriptional and translational processes.

References

- Jacob, W.F. and M.H. Taylor. 1983. The time course of seawater acclimation in *Fundulus heteroclitus* L. J. Exp. Zool. 228: 33-39
- Marshall, W.S., Emberley, T.R., Singer, T.D., Bryson, S.E. and S.D. McCormick. 1999. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. J. Exp. Biol. 202: 1535-1544
- McCormick, S.D. 1993. Methods for non-lethal gill biopsy and measurement of Na^+, K^+ -ATPase activity. Can. J. Fish Aquat. Sci. 50: 656-658
- McCormick, S.D. and H.A. Bern. 1989. *In vitro* stimulation of $\text{Na}^+ - \text{K}^+$ -ATPase activity and ouabain binding by cortisol in coho salmon gill. Am. J. Physiol. 256: R707-R715
- Towle, D.W., Gilman, M.E. and J.D. Hempel. 1977. Rapid modulation of gill $\text{Na}^+ + \text{K}^+$ -dependent ATPase activity during rapid acclimation of the killifish *Fundulus heteroclitus* to salinity change. J. Exp. Zool. 202: 179-186
- Wood, C.M. and W.S. Marshall. 1994. Ion balance, acid-base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus*- A euryhaline estuarine teleost. Estuaries 17: 34-52

REGULATION OF IONIC AND OSMOTIC BALANCE
DURING SMOLTING AND DESMOLTING OF HATCHERY-REARED
SAIMAA LANDLOCKED SALMON (*Salmo salar m. sebago* Girard)

Päivi Kiiskinen
Department of Biology, University of Joensuu
P.O.Box 111, FIN-80101 Joensuu, Finland
Phone: +358 13 2514691, Fax: +358 13 2513590
E-mail: paivi.kiiskinen@joensuu.fi

Heikki Hyvärinen¹, Jorma Piironen²
¹Department of Biology, University of Joensuu
P.O.Box 111, FIN-80101 Joensuu, Finland
²Finnish Game and Fisheries Institute,
Saimaa Fisheries Research and Aquaculture
FIN-58175 Enonkoski, Finland

EXTENDED ABSTRACT ONLY - DO NOT CITE

Saimaa landlocked salmon (*Salmo salar m. sebago* Girard) has been isolated in fresh water at least for several thousands of years as a consequence of the isostatic uplift of the landmass following the last ice age. Under natural conditions this salmon displays a life pattern similar to those of their anadromous conspecifics. However, the construction of hydroelectric dams in the middle of the 1950's and early 1970's destroyed all natural breeding areas. Hence, the present stock of endangered Saimaa landlocked salmon is maintained by stocking hatchery-reared smolts near to their previous reproduction areas. This necessitates detailed studies of parr-smolt transformation under fish farm conditions.

Physiological changes associated with smolting and desmolting were examined in one- and two-year-old hatchery-reared Saimaa landlocked salmon. The early development of the fish was accelerated by increasing the water temperature from 4 to 8°C from about four weeks after the eyed stage of the embryos until startfeeding when the ambient temperature reached 8°C. Studies were done in

one-year-old upper modal and two-year-old lower modal fish as well as in fish which were not graded into upper and lower modal groups within year classes. For determination of the smolting stage, the development of external smolt indices, size and condition factor, muscle lipid content, gill Na^+, K^+ -ATPase activity and plasma ion concentrations (Na^+ , Cl^- and Mg^{2+}) were measured from ten randomly selected fish from each group at intervals of about one month in January-July or April-December. In addition, ten to twenty fish from each group were exposed to a seawater challenge test (SW test) for 48 h at c. 30 ‰. Tests were done at constant 10°C temperature. The mortality, muscle water content and concentrations of plasma ions (Na^+ , Cl^- and Mg^{2+}) were determined from these test fish.

Our results from studies with hatchery reared Saimaa landlocked salmon showed that transformation processes associated with smolting are similar to those of anadromous Atlantic salmon (*Salmo salar*) including typical smolt colouration, reduced condition factor, decreased muscle lipid content and elevated gill Na^+, K^+ -ATPase activity and hypoosmoregulatory ability. Based on these parameters smolt status was reached during April-May and May-June in two- and one-year-old fish, respectively. Interestingly, a transitory reduction in freshwater osmoregulatory capacity was observed coincident with smolting as judged by decreased plasma ion concentrations (Na^+ , Cl^- and Mg^{2+}) and high muscle water content in fresh water. Desmolting was observed after June-July as indicated by darkening of the scales, increases in condition factor and muscle lipid content and decrease in gill Na^+, K^+ -ATPase activity. However, no clear reduction in hypoosmoregulatory ability was observed, but the capacity to regulate the osmotic and ionic balance in seawater was maintained until late autumn.

The current research is concentrated on clarifying seasonal changes in structure of gill chloride cells as well as gill Na^+, K^+ -ATPase activity and plasma ion (Na^+ , Cl^- and Mg^{2+}) concentrations in fish exposed to seawater challenge test for 48-96 h at ambient and 10°C temperatures. Regulation of osmotic and ionic balance during smolting and desmolting as well as idea of smolting as evidence of maladaptation of the fish to fresh water will be discussed.

**CHANGES IN GILL ION TRANSPORT PROTEIN EXPRESSION
ASSOCIATED WITH ENVIRONMENTAL SALINITY. A TALE FROM
THE COHO SALMON (*ONCORHYNCHUS KISUTCH*)**

Jonathan M. Wilson,
Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR),
Universidade do Porto, Rua do Campo Alegre 823
4150-180 Porto, Portugal
Ph (351) 22 606 0421 Fax (351) 22 606 0423
Email: wilson_jm@cimar.org

A. Wayne Vogl¹, Bruce L. Tufts² and David J. Randall³
¹ Department of Anatomy, University of British Columbia, Canada
² Department of Biology, Queens University, Kingston, Canada
³ Department of Zoology, University of British Columbia, Canada V6T 1Z4.

EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

The ionic regulatory needs of teleost fishes living in fresh water and sea water are diametrically opposed so animals moving between these environments are presented with a physiologically challenging situation. These fishes must realign their transepithelial ion transport mechanisms to successfully adapt (ion uptake in freshwater and ion elimination in seawater). A number of salmonid species make these migrations and have thus been a popular group to study from both academic and commercial perspectives. Ion transport proteins (ATPases, symporters, antiporters and channels) and paracellular pathways form the basis of transepithelial ion movements. In the present paper, we will focus on the distribution of Na⁺,K⁺-ATPase, Cl⁻/HCO₃⁻ anion exchanger (AE), and Na⁺/H⁺ exchanger in the gills (and gut) of juvenile coho salmon (*Oncorhynchus kisutch*) which have been raised in fresh water or transferred to sea water. Of interest is the fate of the freshwater chloride cell (Cl⁻ uptake utilizing an apical AE; Goss et al. 1995) in fish transferred to sea water and NHE 2-like protein which appears to label the accessory cell type which is associated with NaCl elimination. (Wilson et al. 2000)

Materials and Methods

Juvenile coho salmon (*O. kisutch*) raised in freshwater or acclimated to seawater for 6 weeks were obtained from the Department of Fisheries and Oceans West Vancouver facility. Six animals were sampled from each group and gill and intestinal tissue fixed in Bouin's solution and embedded in paraffin. Ion transport proteins were localized using indirect immunofluorescence techniques employing non-homologous antibodies (Table 1).

Table 1. List of antibodies employed and their sources

Antibody	Antigen	Host	Source
Na ⁺ ,K ⁺ -ATPase	α-subunit	mouse	DSHB*
Na ⁺ /H ⁺ Exchanger	NHE-2 fusion protein	rabbit	Hoogerwerf et al. 96
Anion Exchanger	trout erythrocyte AE1	rabbit	Cameron et al. 96

*Developmental Studies Hybridoma Bank, University of Iowa, USA

Results and Discussion

Anion Exchanger (AE)

In freshwater coho salmon, the branchial epithelium has a typical teleostean population of mitochondria-rich (MR) cells which show high levels of Na⁺,K⁺-ATPase immunoreactivity (Fig1B). A sub-population of these MR cells in the lamellar epithelium show apical immunoreactivity for the AE1 (arrows; Fig1A) in addition to erythrocytes, which show strong immunoreactivity (Fig1A,D). The AE labeled freshwater MR cells are presumably active in freshwater Cl⁻ uptake (= Chloride Cells; apical Cl⁻/HCO₃⁻ anion exchanger; Goss et al. 1995, Wilson et al. 2000).

In coho salmon that have been acclimated to sea water, there is an absence of AE1 apical immunoreactivity (Fig1D) associated with MR cells (identified by high Na⁺,K⁺-ATPase immunoreactivity) (Fig1E). Predictably, the freshwater Cl⁻ uptake cells are lost in the seawater acclimated fishes. Apical AE1 immunoreactivity is, however, observed in enterocytes of seawater acclimated animals (data not shown). These intestinal cells are presumably involved in the HCO₃⁻ elimination reported by R.W. Wilson et al. 1996.

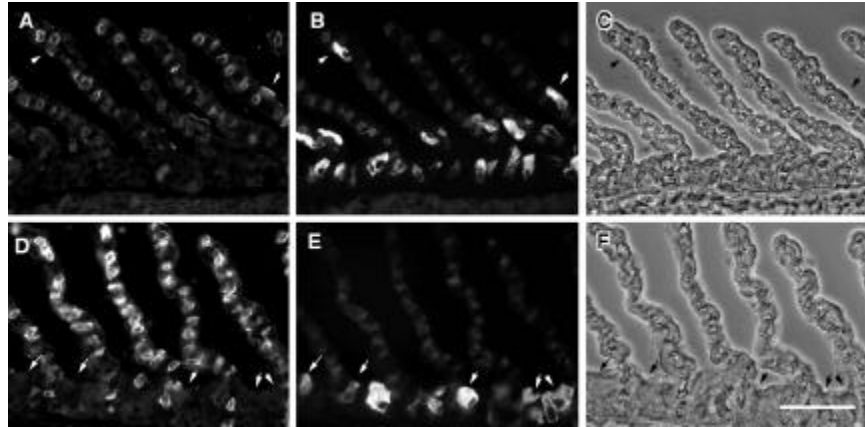


Figure 1 Immunohistochemistry of double labeled sections from the gills of coho salmon adapted to either freshwater (**ABC**) or seawater (**DEF**) showing the distributions of the band 3-like anion exchanger (AE1;**A,D**) and Na^+,K^+ -ATPase (**B,E**). The corresponding phase contrast images are shown for orientation (**C** and **F**, respectively). Scale bar = 50 μm .

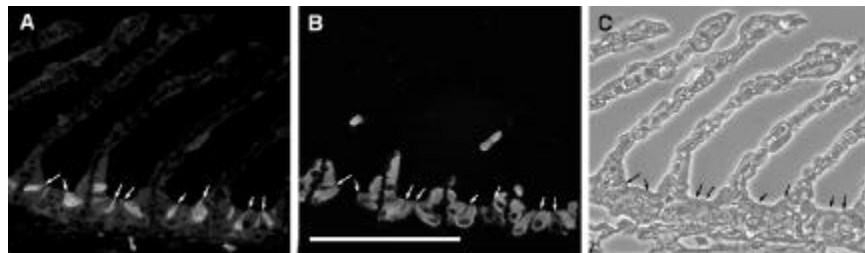


Figure 2 Double labeled section of the seawater adapted coho gill epithelium using a rabbit polyclonal antibody (597) against the NHE-2 (*arrows*; **A**) and mouse monoclonal antibody against the α subunit of Na^+,K^+ -ATPase (**B**). The corresponding phase contrast image is shown in (**C**). Scale Bar= 50 μm

Na^+/H^+ Exchanger (NHE)

NHE 2-like immunoreactivity is associated with cells in the interlamellar spaces that do not have high Na^+,K^+ -ATPase immunoreactivity (Fig2). These NHE-2 labeled cells appear to be accessory cells on the basis of their morphology and location. Although typically associated with NaCl elimination in seawater teleost fishes, these cells are also found in the freshwater fish sampled. These

results are consistent with the literature (Pisam and Rambourg 1991). However, the functional significance of the accessory cell labeling is not clear, although, in any case, the NHE-2 antibody may prove to be a useful marker for the accessory cell type in future studies.

Acknowledgements

We would like to thank Bob Devlin for kindly supplying the fishes, Mark Donowitz for the NHE antisera and NSERC for the funding.

References

- Cameron, B.A., S.F. Perry, C. Wu, K. Ko, and B.L. Tufts 1996 Bicarbonate permeability and immunological evidence for an anion exchanger-like protein in the red blood cells of the sea lamprey, *Petromyzon marinus*. *J. Comp. Physiol.*, 166:197-204.
- Goss, G.G., S.F. Perry and P. Laurent. 1995. Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. In *Cellular and Molecular Approaches to Fish Ionic Regulation*. T.W. Shuttleworth C.M. Wood, pp. 257-284. San Diego: Academic Press.
- Hoogerwerf, W.A., S.C. Tsao, O. Devuyst, S.A. Levine, C.H. Yun, J.W. Yip, M.E. Cohen, P.D. Wilson, A.J. Lazenby, C.M. Tse, and M. Donowitz 1996 NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. *Am. J. Physiol.* 270:G29-G41
- Pisam, M. and A. Rambourg 1991 Mitochondria-rich cells in the gill epithelium of teleost fishes: An ultrastructural approach. *Inter.Rev.Cytol.* 130:191-232.
- Wilson, J.M., P. Laurent, B.L. Tufts, M. Donowitz, D.J. Benos, A.W. Vogl, and D.J. Randall 2000 NaCl uptake in freshwater fishes. An immunological approach to ion transport protein localization. *J. Exp. Biol.* (in press)
- Wilson, R.W., K.M. Gilmour, R.P. Henry, and C.M. Wood. 1996. Intestinal base excretion in the seawater-adapted rainbow trout: A role in acid-base regulation? *J. Exp. Biol.* 199: 2331-2343.

**LOCALIZATION AND CHANGES IN THE ABUNDANCE
OF THE Na⁺-K⁺-2Cl⁻ COTRANSPORTER DURING
SMOLTING, SALINITY ACCLIMATION AND
FOLLOWING HORMONE TREATMENT**

Ryan M. Pelis
Conte Anadromous Fish Research Center, BRD/USGS,
P.O. Box 796, Turners Falls, MA, 04376, USA and
Organismic and Evolutionary Biology, University of Massachusetts,
Amherst, MA 01003, USA.
Phone: 413-863-3833 E-mail: Rpelis@nsm.umass.edu

Joseph Zydlewski
Conte Anadromous Fish Research Center, USGS/BRD

Stephen D. McCormick
Conte Anadromous Fish Research Center, USGS/BRD

EXTENDED ABSTRACT ONLY – DO NOT CITE

Depending on the salinity of the surrounding environment, gill chloride cells of euryhaline teleosts may vary in respect to the quantity and types of membrane transporters they contain. While the Na⁺-K⁺-2Cl⁻ cotransporter has never been localized to chloride cells, it has been suggested to be present in chloride cells of teleosts based on the ability of loop diuretics to inhibit the short circuit current generated by opercular epithelia (Eriksson et al. 1985). Unlike Na⁺,K⁺-ATPase, our current models of chloride cells from fresh water and seawater adapted teleosts suggests that the Na⁺-K⁺-2Cl⁻ cotransporter is only present in cells from seawater-adapted fish.

In Atlantic salmon, smolting and acquisition of salinity tolerance is under endocrine control. In particular, growth hormone and cortisol have been found to increase gill Na⁺,K⁺ATPase activity and salinity tolerance in Atlantic salmon

(McCormick 1996). While other hormones may also be important for increased survival during seawater acclimation, these hormones are likely necessary. This study was conducted in order to determine whether the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter is present in chloride cells and how its localization and expression are regulated during smolting, salinity acclimation and following growth hormone and cortisol treatment.

Immunocytochemistry and immunoblotting procedures were performed in order to localize and quantify the amounts of the cotransporter present within the gill epithelium of Atlantic salmon. A monoclonal antibody that recognizes the secretory and absorptive isoforms of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (Lytle et al. 1995) was used as a primary antibody for immunocytochemistry and immunoblots. In order to determine whether the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter is present within chloride cells it was colocalized with $\text{Na}^+\text{,K}^+\text{-ATPase}$ through the use of a rabbit polyclonal antibody directed against the α -subunit of $\text{Na}^+\text{,K}^+\text{-ATPase}$ (Ura et al. 1996).

The $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter was localized to chloride cells and not other cells in the gill of Atlantic salmon. In parr, only a few chloride cells stained positive for the cotransporter and these cells appeared on both the primary filaments and secondary lamellae. Acclimation of parr to 30 ppt seawater increased the number of large spherical immunoreactive chloride cells on primary filaments, whereas few cells were stained on the secondary lamellae. Immunoblots indicated that parr acclimated to 30 ppt seawater contained more of the cotransporter than freshwater acclimated parr.

Smolts in May exhibited larger numbers of immunoreactive cells on primary filaments and secondary lamellae over levels displayed in pre-smolts in February, post-smolts in June and parr in May. $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter abundance increased significantly between February and May and declined to levels found in parr by June. Changes in the abundance of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter were similar to that of gill $\text{Na}^+\text{,K}^+\text{-ATPase}$ activity ($\mu\text{moles ADP/mg protein/hr}$) before, during and after smolting.

Growth hormone (GH; 5 $\mu\text{g/g}$), cortisol (F; 50 $\mu\text{g/g}$) and growth hormone + cortisol (GH + F) treatments were administered to Atlantic salmon parr to determine if these hormones regulate the localization and expression of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. F and GH + F treatments resulted in greater numbers of immunoreactive chloride cells (primary filament and secondary lamellar; $\text{cc}/\mu\text{m}$)

over levels seen in vehicles. The number of immunoreactive chloride cells on the primary filament ($\text{cc}/\mu\text{m}$) were increased by all three treatments with the greatest affect amongst the F and GH + F treated fish. Primary filament immunoreactive chloride cell area ($\mu\text{m}^2/\text{cell}$) was increased by all three treatments with the greatest affect in the GH + F treated fish. The shape factor of primary filament immunoreactive chloride cells was increased with GH + F treatment only, which indicates that this treatment resulted in the cells becoming more circular in shape. The number ($\text{cc}/\mu\text{m}$), size ($\mu\text{m}^2/\text{cell}$) and shape factor of secondary lamellar immunoreactive chloride cells was not significantly affected by the three treatments. Gill Na^+, K^+ -ATPase activity was elevated by F and GH + F treatment only. Immunoblots indicated that all three treatments (GH, F and GH + F) resulted in greater levels of the cotransporter compared to the vehicle with each of the treatments being different as well (GH + F > F > GH).

In summary, the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter is located in gill chloride cells of Atlantic salmon and is upregulated during smolting and salinity acclimation. Smolting and salinity acclimation also resulted in changes in the localization of the protein; seawater-adapted fish expressed the protein in chloride cells along the primary filament while smolts expressed the protein in chloride cells on the primary filament and secondary lamellae. Cortisol and growth hormone treatment alone increased cotransporter abundance, while both hormones together had an even greater affect. All three treatments increased the number and size of primary filament immunoreactive chloride cells with GH + F treatment having the greatest affect on size. Primary chloride cell shape was affected by GH + F treatment only. The hormones (growth hormone and cortisol), which are important for smolting and the acquisition of salinity tolerance in Atlantic salmon, and that regulate the expression of Na^+, K^+ -ATPase, are also involved in the regulation of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter.

References

- Eriksson, O., N. Mayer-Gostan, and P. J. Wistrand. 1985. The use of fish opercular epithelium as a model tissue for studying intrinsic activities of loop diuretics. *ActaPhysiol. Scand.* 125: 55-66.
- Lytle, C., J. Xu, D. Biemesderfer, and B. Forbush III. 1995. Distribution and

diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. *Am. J. Physiol.* 269 (Cell Physiol. 38): C1496-C1505.

McCormick, S. D. 1996. Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na⁺,K⁺-ATPase in Atlantic salmon (*Salmo salar*): Interaction with cortisol. *Gen. Comp. Endocrinol.* 101: 3-11.

Ura, K., K. Soyano, N. Omoto, S. Adachi, and K. Yamauchi. 1996. Localization of Na⁺,K⁺-ATPase in tissues of rabbit and teleosts using an antiserum directed against partial sequence of the α -subunit. *Zool. Sci.* 13: 219-227.

**GILL AND INTESTINAL Na^+ - K^+ ATPase ACTIVITY,
AND ESTIMATED OSMOREGULATORY COSTS,
IN HIGH-ENERGY-DEMAND TELEOSTS:
YELLOWFIN TUNA (*Thunnus Albacares*),
SKIPJACK TUNA (*Katsuwonus Pelamis*), AND
DOLPHIN FISH (*Coryphaena Hippurus*)**

Richard Brill

Pelagic Fisheries Research Program, Joint Institute for Marine and
Atmospheric Research, School of Ocean and Earth Science and Technology,
University of Hawaii at Manoa, Honolulu, Hawaii, 96822, U.S.A.
Mailing address: NMFS, 2570 Dole St., Honolulu, Hawaii 96822-2396, U.S.A.,
phone: 808-592-8304, FAX: 808-592-8300, e-mail:
rbrill@honlab.nmfs.hawaii.edu

Yonat Swimmer

Pelagic Fisheries Research Program, Joint Institute for Marine and
Atmospheric Research, School of Ocean and Earth Science and Technology,
University of Hawaii at Manoa, Honolulu, Hawaii, 96822, U.S.A.

EXTENDED ABSTRACT ONLY - DO NO CITE

Skipjack and yellowfin tuna (*Katsuwonus pelamis* and *Thunnus albacares*, respectively) and dolphin fish (*Coryphaena hippurus*) have gill blood-water barriers up to approximately an order of magnitude thinner; and gill surface areas, ventilation volumes, and cardiac outputs from several times to up to almost an order of magnitude greater than those of other teleosts (Perry, 1992; Bushnell and Jones, 1994). As a consequence, tunas (and presumably also dolphin fish) exhibit routine oxygen transfer factors (TO_2 , the rate of O_2 transfer from water to blood per unit partial pressure difference between inhalant water and venous blood) at least an order of magnitude above those of other fishes (Bushnell and Brill, 1992). We propose that the morpho-physiological

adaptations which permit tunas to achieve such exceptional TO_2s , and maximum metabolic rates (MMR) that are several times greater than those of other fishes, should also result in high water and ion flux rates across the gills and concomitant high osmoregulatory costs. In other words, we contend that teleosts which are capable of achieving exceptionally high TO_2s and MMR, necessarily have high standard metabolic rates (SMR) due to elevated rates of energy expenditure required for osmoregulation. Although osmoregulatory costs in tunas and dolphin fish have never been measured, studies have confirmed that these fish all have SMR several times those of other active teleosts (e.g., Brill, 1987). Previous investigators have shown a link between $\text{Na}^+\text{-K}^+$ ATPase activity in the gills and activity patterns by studying epipelagic and sluggish deep-sea fishes (Gibbs and Somero, 1990). Based on these observations, we hypothesize that high-energy-demand fishes (i.e., tunas and dolphin fish) have elevated gill and intestinal $\text{Na}^+\text{-K}^+$ ATPase activities to compensate for the high rates of passive ion and water movements occurring across their exceptionally large, thin gills.

To test this idea and indirectly estimate osmoregulatory costs, we measured $\text{Na}^+\text{-K}^+$ ATPase activity (V_{max}) in homogenates of frozen samples taken from the gills and intestine of skipjack and yellowfin tunas, and the gills dolphin fish. As a check of our procedures, we made similar measurements using gill and intestinal tissue from hybrid red tilapia (*Oreochromis mossambicus* x *O. niloticus*). We also determined gill filament and gut masses so as to be able to calculate the fraction of the SMR attributable to maximal $\text{Na}^+\text{-K}^+$ ATPase activity.

Contrary to our supposition, we found only small difference in $\text{Na}^+\text{-K}^+$ ATPase activity per unit mass of gill tissue in these four species, although $\text{Na}^+\text{-K}^+$ ATPase activity per unit mass of intestinal tissue was higher in tilapia (Table 1). Our results, were, moreover, comparable to values previously reported for tilapia and active marine teleosts .

Table 1.

	gill Na ⁺ -K ⁺ ATPase (mmoles ATP h ⁻¹ g ⁻¹ wet weight)	gill Na ⁺ -K ⁺ ATPase (mmoles ATP h ⁻¹ mg ⁻¹ protein)
yellowfin tuna	245 ± 20, n=13	4.6 ± 0.5, n=14
skipjack tuna	217 ± 36, n=10	4.2 ± 0.8, n=12
dolphin fish	342 ± 39, n=7	9.7 ± 1.1, n=12
tilapia	341 ± 41, n=7	7.3 ± 0.4, n=13
yellowfin tuna	165 ± 16, n=14	3.4 ± 0.4, n=15
skipjack tuna	227 ± 41, n=10	4.9 ± 0.6, n=10
tilapia	439 ± 42, n=7	5.2 ± 0.5, n=7

Based on gill and intestinal Na⁺-K⁺ ATPase activity, we estimate the cost of osmoregulation to be at most 13 %, and 9 %, of the SMR in yellowfin and skipjack tuna (respectively), and 63% hybrid red tilapia. Our results, therefore, do not support our original suppositions. Rather, we conclude: (1) rates of energy expenditure required for counteracting the passive influx of ions and the water loss occurring across the gills of tunas and dolphin fish are not exceptional when expressed as a fraction of the SMR; and (2) osmoregulatory costs are not responsible for the elevated SMR of tunas and dolphin fish.

References

- Brill, R. W. 1987. On the standard metabolic rates of tropical tunas, including the effect of body size and acute temperature change. *Fish. Bull. U.S.* 85: 25-35.
- Bushnell, P. G. and R. W. Brill. 1992. Oxygen transport and cardiovascular responses in skipjack tuna (*Katsuwonus pelamis*) and yellowfin tuna

(*Thunnus albacares*) exposed to acute hypoxia. J. Comp. Physiol. B 162: 131-143.

Bushnell, P. G. and D. R. Jones. 1994. Cardiovascular and respiratory physiology of tuna: adaptations for support of exceptionally high metabolic rates. Environ Biol Fishes 40:303-318.

Gibbs A, Somero GN (1990) Na^+ - K^+ -adenosine triphosphate activities in gills of marine teleost fishes: changes with depth, size and locomotory activity. Mar. Biol. 106:315-321.

Perry, S. F. 1992. Morphometry of vertebrate gills and lungs: a critical review. In S. Egginton and H. F. Ross (eds) Oxygen Transport in Biological Systems: Modeling of pathways from environment to cell. Society for Experimental Biology Seminar Series 51. Cambridge University Press, Cambridge, pp 57-77.

Acknowledgments

This paper was funded by Cooperative Agreements NA37RJ0199 and NA67RJ0154 from the National Oceanic and Atmospheric Administration (NOAA) with the Joint Institute for Marine and Atmospheric Research, University of Hawaii; and by the National Marine Fisheries Service (Southwest Fisheries Science Center, Honolulu Laboratory).

**A MOLECULAR INVESTIGATION OF THE ROLE OF THE
BRANCHIAL VACUOLAR H⁺-ATPASE IN ACID-BASE BALANCE
AND IONIC REGULATION IN RAINBOW TROUT
(*ONCORHYNCHUS MYKISS*)**

S. F. Perry, C. Dumont and D. A. Johnson
Department of Biology, University of Ottawa, 30 Marie Curie,

Ottawa, Ontario, Canada K1N 6N5

Phone (613) 562-5800 x6005 Fax (613) 562-5486

E-mail sfperry@science.uottawa.ca

Key words: Vacuolar H⁺-ATPase, proton pump, V-type ATPase, gill, fish, hypercapnia, cortisol, softwater

Abstract

A cloned cDNA fragment of the rainbow trout gill vacuolar H⁺-ATPase (H⁺V-ATPase; proton pump) B subunit was used as a probe to examine i) its inter-specific distribution among marine and freshwater species and ii) its expression during a variety of acid-base and ionic disturbances. Northern blots of gill total RNA, performed under conditions of high stringency, revealed cross hybridisation between the trout probe and 11 of 16 species that were examined. Cross hybridisation was not observed in the pacific hagfish (*Eptatretus stoutii*), Lake Magadi tilapia (*Oreochromis alcalicus Grahami*), bigfin eelpout (*Lycodes cortezianus*), blackfin poacher (*Bathyagonus nigripinnus*) or freshwater American eel (*Anguilla rostrata*).

Acute (3 h) exposure of trout to external hypercapnia (PwCO₂ = ~7 mm Hg) was associated with a transient increase after 1 h in gill H⁺-ATPase mRNA levels. Thus, the increase in gill H⁺-ATPase activity that is known to accompany hypercapnic acidosis in trout (Lin and Randall 1993) may reflect, at least in part, its transcriptional or post-transcriptional regulation. Plasma cortisol levels were elevated in the hypercapnic fish (from 45 ± 15 to 83 ± 4 ng ml⁻¹) and because

cortisol was previously implicated as a regulator of H⁺-ATPase activity (Lin and Randall 1993), mRNA levels were quantified in fish subjected to chronic cortisol elevation. An increase in plasma cortisol concentration from 90 ± 10 (sham implants) to 300 ± 60 ng ml⁻¹ (cortisol implants) for 4 days was associated with an approximate doubling of gill H⁺-ATPase steady-state mRNA levels.

Exposure of trout for 72 h to ion-poor water caused a persistent reduction in the concentration of gill H⁺-ATPase steady-state mRNA. The functional significance of this response is unclear but may reflect a reduced rate of Na⁺ uptake across the gill. These results are discussed with reference to the physiological role of the branchial H⁺-ATPase in both acid-base and ionic regulation.

Introduction

The vacuolar H⁺-ATPase (H⁺V-ATPase) or proton pump is probably expressed in all eukaryote cells where it plays a housekeeping role in the acidification of intracellular organelles (Nelson 1992). However, in addition to its housekeeping role, the H⁺V-ATPase is thought to be specifically involved in acid-base balance and ionic regulation in a variety of secretory epithelia (see reviews by Stevens and Forgac 1997; Forgac 1998; Nelson and Harvey 1999; Wiczorek et al. 1999) including the rainbow trout (*Oncorhynchus mykiss*) gill (Lin and Randall 1995). Avella and Bornancin (1989) first suggested a physiological role for the H⁺V-ATPase in the fish gill. Specifically, it was reasoned using thermodynamic arguments that the traditional model for Na⁺ uptake across the freshwater fish gill, involving electroneutral Na⁺/H⁺ exchange (Krogh 1938), was not tenable. Instead, it was postulated (Avella and Bornancin, 1989) that Na⁺ uptake across the apical membrane of gill epithelial cells was linked energetically to active H⁺ extrusion via the H⁺V-ATPase. According to their model, H⁺ secretion across the apical membrane establishes a favourable electrochemical gradient that permits the inward entry of Na⁺ through epithelial Na⁺ channels.

Despite scarce empirical evidence for apical membrane Na⁺ channels, this newer model for Na⁺ uptake is now generally accepted (Marshall 1995). Less certain, however, is the epithelial location of the fish gill the H⁺V-ATPase. Indeed, arguments have been made for a specific localisation to the chloride cell (Lin and Randall 1991), the pavement cell (Laurent *et al.* 1994; Sullivan *et al.* 1995; Kultz and Somero 1995; Sullivan, *et al.* 1996) or both cell types (Lin *et al.* 1994;

Lin and Randall 1995; Perry 1997). Regardless of its location, evidence is accruing that the activity of the H⁺V-ATPase in the fish gill is regulated in accordance with acid-base and ionic uptake requirements (Lin and Randall 1995; Perry and Fryer 1997). For example, H⁺V-ATPase activity is increased in the trout gill during respiratory acidosis (Lin and Randall 1993; Sullivan *et al.* 1995) and decreased during seawater exposure (Lin and Randall 1993). Because cortisol (Lin and Randall 1993) and growth hormone (Perry and Fryer 1997) are known to influence branchial H⁺V-ATPase activity, the reported changes in its activity during environmental changes may reflect hormonal regulation. Changes in H⁺V-ATPase activity could reflect transcriptional or post-transcriptional regulation of mRNA levels and/or translational or post-translational regulation of H⁺V-ATPase protein activity/localisation. Sullivan *et al.* (1996) provided evidence in support of transcriptional/post-transcriptional regulation by demonstrating, using *in situ* hybridisation, that the levels of H⁺V-ATPase mRNA were increased during exposure of rainbow trout to external hypercapnia. That study, however, was limited by the use of a heterologous oligonucleotide probe that was derived from the sequence of bovine renal H⁺V-ATPase E subunit. Recently, the B sub-unit of the H⁺V-ATPase was cloned from trout gill (Perry *et al.* 2000) and thus an homologous cDNA probe is now available to quantitatively assess the levels of H⁺V-ATPase mRNA.

In this study, we have used the previously cloned H⁺V-ATPase cDNA to i) establish its inter-specific distribution among marine and freshwater species using northern analysis and ii) assess its expression in rainbow trout gill during a variety of acid-base, ionic and hormonal disturbances using slot blot analysis.

Materials and Methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 200-300 g, < 2 years old) of both sexes were obtained from Linwood Acres Trout Farm. American eels, *Anguilla rostrata*, of either sex (~ 500 g) and brown bullhead catfish, *Ictalurus nebulosus*, (~ 300 g) were obtained from a commercial supplier (Lancaster Ontario) and were transported on ice (eels) or in oxygenated water (bullhead) to University of Ottawa. All species were held indoors (using a 12:12-h light-dark photoperiod cycle) in large 1300 l fiberglass tanks supplied with flowing, aerated, and dechlorinated tap water. Trout and eel were maintained at a

temperature of 14°C, bullhead were kept at 20° C. While trout and bullhead were fed commercial diets *ad libitum* on alternate days, eels were not fed.

In addition, total RNA was obtained (courtesy of Dr. Pat Walsh, University of Miami) from the following species; winter flounder (*Pseudopleuronectes americanus*), Pacific hagfish (*Eptatretus stoutii*), lingcod (*Ophiodon elongatus*), long-horned sculpin (*Myoxocephalus octodecimspinosus*), Lake Magadi tilapia (*Oreochromis alcalicus Grahami*), plainfin midshipman (*Porichthys notatus*), bigfin eelpout (*Lycodes cortezianus*), Dover sole (*Microstomus pacificus*), shiner perch (*Cymatogaster aggregata*), green cod (*Triglops macellus*), gulf toadfish (*Opsanus beta*), brown Irish lord (*Hemilepidotus spinosus*), black finned poacher (*Bathyagonus nigripinnus*) and lumpfish (*Cyclopterus lumpus*).

Experimental protocol

In addition to a broad species survey and an assessment of H⁺V-ATPase tissue distribution in rainbow trout, three separate series of experiments were performed.

1. Exposure to external hypercapnia

To induce rapid respiratory acidosis, separate groups of fish (N = 6 in each group) were exposed to external hypercapnia for 1, 2 or 3 h. Hypercapnia was achieved by gassing a water equilibration column with 1.3% CO₂ in air (Cameron flowmeter) to reach a final water PCO₂ (P_wCO₂) of ~7.5 mm Hg. The P_wCO₂ was monitored continuously using a CO₂ electrode and associated meter (Cameron Instruments Inc.). Deviations in P_wCO₂ from the target of 7.5 mm Hg were corrected by adjustments of gas and/or water flows through the equilibration column. Control fish were subjected to continuing normocapnia for 1- 3 h. At 0, 1, 2, and 3 h, fish were euthanised and perfused with saline to remove blood from the tissues (Perry *et al.* 2000). Tissues were removed, frozen in liquid N₂ and stored at -80° C until total RNA was prepared.

2. Exposure to ion-poor water

Ion-poor water was prepared by mixing tap water with deionised water derived from a reverse osmosis unit. Regular tap water and the ion-poor water were analysed daily for Na⁺, Ca²⁺ and K⁺ levels using flame emission spectroscopy (Varian model 250 atomic absorption spectrophotometer). For regular tap

water, the ion concentrations (in mmol l⁻¹) were Na⁺ = 0.135, Ca²⁺ = 0.391 and K⁺ = 0.025. For ion-poor water, the ion concentrations (in mmol l⁻¹) were Na⁺ = 0.03 - 0.06, Ca²⁺ = 0.09 - 0.13 and K⁺ = 0.006 - 0.009. Fish were not fed during the duration (6 - 72 h) of the ion-poor water treatment. Fish were sacrificed (N = 6 in each group) after 6, 12, 24, 48 and 72 h of exposure to ion-poor water.

3. Cortisol treatment

Fish were anaesthetised in a 1:12,000 (weight/volume) solution of benzocaine (ethyl-*p*-aminobenzoate) cooled to 10° C. After cessation of breathing movements, the fish was transferred to an operating table and the gills were irrigated with the same anaesthetic solution throughout the brief period (< 1 min) required to inject cortisol implants. To permit chronic elevation of circulating cortisol levels to a target level of 200 - 300 ng ml⁻¹, fish were injected intraperitoneally with 150 mg kg⁻¹ body weight (injection volume = 2 ml kg⁻¹) of cortisol (hemisuccinate salt) dissolved in coconut oil (Perry and Reid 1994). Control fish were injected with equivalent volumes of coconut oil. Control and cortisol-treated fish were kept in separate tanks (100 L) for 4 days prior to blood sampling and tissue removal (see below).

Fish were killed by a blow to the head and a blood sample (~ 1 ml) was rapidly withdrawn from the caudal vessels by percutaneous puncture. After centrifugation (30 sec at 12,000 G), plasma was removed and frozen (-20° C) for subsequent determination of cortisol levels. Tissues were harvested and immediately frozen in liquid nitrogen prior to storage at -80° C.

RNA isolation, gel electrophoresis and Northern analysis

Total RNA was isolated by homogenisation in guanidinium thiocyanate (Chomczynski and Sacchi, 1987) as modified by Chomczynski and Mackey (1995).

Northern analysis was performed using an 810 base pair homologous H⁺V-ATPase cDNA plasmid insert as the probe (Perry *et al.* 2000). RNA samples (20 µg) were incubated in loading buffer at 65° C and electrophoresed through 1.5% (w/v) agarose gels in MOPS (morpholinopropanesulfonic acid) buffer containing 0.6 mol l⁻¹ formaldehyde as described in Sambrook *et al.* (1989), and then transferred to GeneScreen⁺ membranes (NEN Life Sciences) by capillary action (Sambrook *et al.* 1989). Membranes were pre-hybridised at 65° C for 2 - 4

h in a buffer containing 6 X SSC (0.9 mol l⁻¹ NaCl, 0.09 mol l⁻¹ sodium citrate, pH 7.0), 5 X Denhardt's (1 X Denhardt's is 0.1% Ficoll 400,000, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 µg ml⁻¹ single-stranded herring sperm DNA, 1% sodium dodecyl sulfate (SDS) and 10% dextran sulfate. After the addition of probe to a concentration of 1 - 2 x 10⁶cpm ml⁻¹, hybridisation proceeded for 16 h at 65° C in the same solution. Following hybridisation, the membranes were washed several times at 65° C with 0.1 X SSC, 0.1% SDS and exposed to BioMax film plus intensifying screen (Kodak) at -80° C for up to 4 days.

In order to confirm equal loading between samples, membranes were re-probed with a homologous β-actin probe (a 514 bp PCR fragment corresponding to 63-576 of the trout β-actin sequence; GenBank accession #AF254414) under similar conditions but with an exposure time of hours.

DNA for probes was labelled with [- ³²P] dCTP (Amersham, 370 MBq ml⁻¹) by the random-primer method (Feinberg and Vogelstein, 1983). Unincorporated nucleotides were removed by spin columns (Sambrook *et al.* 1989). Prior to use, the labelled DNA was denatured by boiling for 5 min and then quick chilled in ice.

Slot blot analysis

A slot blot apparatus (Schleicher and Schuell, Minifold II) was used for semi-quantitative analysis of H⁺V-ATPase mRNA levels in fish exposed to (treated with) hypercapnia, ion-poor water or cortisol. A preliminary series of experiments revealed that the ratio of H⁺V-ATPase/β-actin phosphorescence was constant only over a relatively narrow range of total RNA loaded (0.5 – 2.0 µg). Consequently, all subsequent slot blot experiments used loading quantities of 0.5 and 1.0 µg total RNA; samples were loaded in duplicate. Thus, for any given sample, an average H⁺V-ATPase/β-actin phosphorescence ratio was obtained using the mean data from both loadings.

After hybridisation and washing (see above), membranes were exposed to a phosphor screen for > 20 h. The screens were subsequently scanned (BioRad Molecular Imager FX) and the signals were quantified using associated BioRad software. Replicate membranes were probed in a similar manner with radiolabelled β-actin but were exposed to the phosphor screens for 30 – 45 min.

Cortisol analysis

Plasma cortisol concentrations were measured on duplicate samples using a commercial radioimmunoassay kit (ICN).

Statistical analysis

All data are represented as means \pm 1 SEM unless otherwise stated. Data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison. P values $<$ 0.05 were considered to be statistically significant. Calculations were performed using the SigmaStat (SPSS; version 2.03) software package.

Results and Discussion

Northern blots of gill total RNA, performed under conditions of high stringency, revealed cross hybridisation between the trout probe and 11 of 16 species that were examined. Cross hybridisation was not observed in the pacific hagfish (*Eptatretus stoutii*), Lake Magadi tilapia (*Oreochromis alcalicus Grahmi*), bigfin eelpout (*Lycodes cortezianus*), blackfin poacher (*Bathyagonus nigripinnus*) or freshwater American eel (*Anguilla rostrata*). A representative Northern blot showing distribution of the H⁺V-ATPase amongst selected fish species is depicted in Figure 1. The lack of detectable cross hybridisation in several of the species cannot be explained by inadequate RNA loading (based on the more-or-less equivalent intensities of the 18 and 28S ribosomal RNA bands; data not shown) but may reflect insufficient sequence homology given the

stringent hybridisation conditions that were used. For example, the trout gill cDNA probe that was used in this study shares 83 – 85% nucleotide identity with similar regions of the eel swim bladder gas gland tissue H⁺V-ATPase B2 and B1 isoforms, respectively (Niederstaetter and Pelster, 2000). It is unclear as to whether this dissimilarity in nucleotide sequence would prevent hybridisation under stringent conditions.

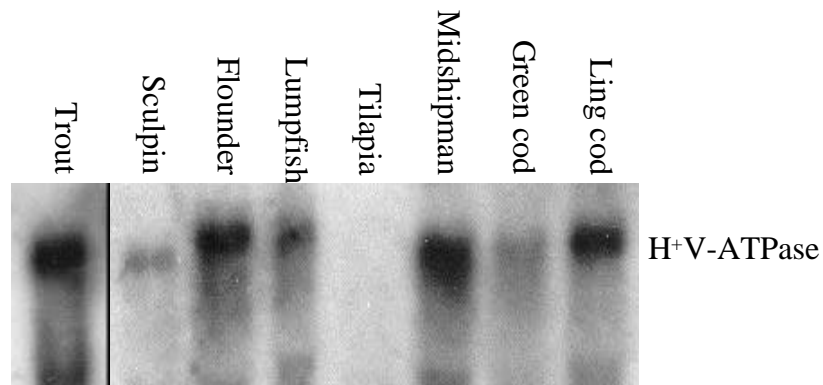


Figure 1. Representative Northern blot of gill tissue from selected fish species showing the degree of cross hybridisation of vacuolar H⁺V-ATPase B subunit mRNA. Transfers were hybridized with a trout H⁺V-ATPase B subunit cDNA probe (810 bp). Gels were loaded with 20 µg per lane of total RNA obtained from individual fish.

A representative Northern blot depicting tissue distribution of H⁺V-ATPase B subunit mRNA is illustrated in Figure 2. Detectable levels of H⁺-ATPase B subunit mRNA were observed in all tissues that were examined. H⁺V-ATPase mRNA expression was high in the gill, kidney (anterior or posterior), intestine, heart, spleen and blood but lower in liver and white muscle.

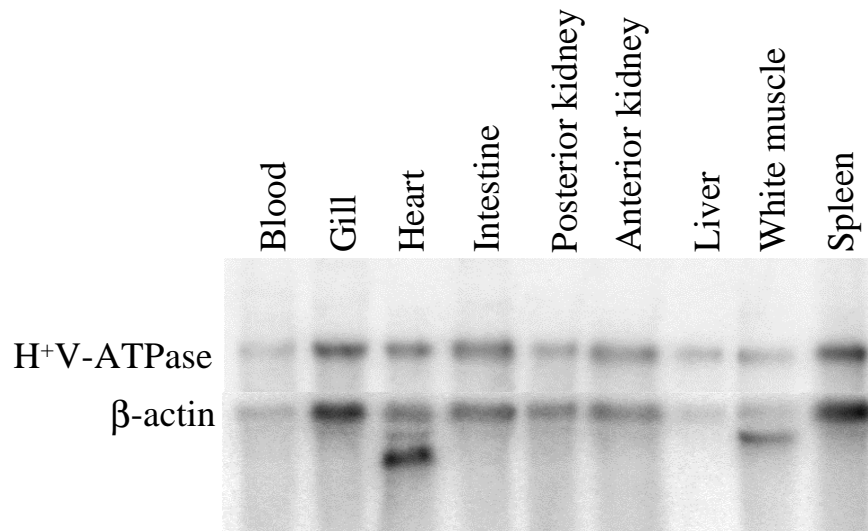


Figure 2. Northern blot of RNA isolated from selected rainbow trout (*Oncorhynchus mykiss*) tissues hybridized with a homologous H⁺V-ATPase B subunit cDNA probe (810 base pairs) and/or a trout β-actin clone. Gels were loaded with 20 μg per lane of total RNA obtained from perfused fish (i.e. blood was removed from tissue via saline perfusion).

Exposure of rainbow trout to external hypercapnia caused a transient elevation of gill H⁺V-ATPase mRNA levels at 1 h (Figure 3). These results are consistent with data from a preliminary study (Perry *et al.* 2000) that demonstrated a trend toward elevated gill H⁺V-ATPase mRNA levels after 2 h of hypercapnia. It is

likely, therefore, that the increased activity of branchial H⁺V-ATPase observed in trout during hypercapnia (Lin and Randall 1993; Sullivan *et al.* 1995) is caused, at least in part, by an increase in the steady-state levels of mRNA. The most probable cause of the elevated mRNA levels is an increase in gene transcription. The physiological benefit of increased branchial H⁺V-ATPase activity during hypercapnia is an associated increase in acid excretion by the gill (Sullivan *et al.* 1995) that serves to raise internal pH and thus correct the hypercapnic acidosis. Although debated, the cell type that is responsible for increased H⁺ excretion during respiratory acidosis excretion is probably the pavement cell rather than the chloride cell (Goss *et al.* 1992; 1995; 1998; Laurent *et al.* 1994).

Plasma cortisol levels were elevated in the hypercapnic fish (from 45 ± 15 to 83 ± 4 ng ml⁻¹) and because cortisol was previously implicated as a regulator of H⁺-ATPase activity (Lin and Randall 1993), mRNA levels were quantified in fish subjected to chronic cortisol elevation. An increase in plasma cortisol concentration from 90 ± 10 (sham implants) to 300 ± 60 ng ml⁻¹ (cortisol

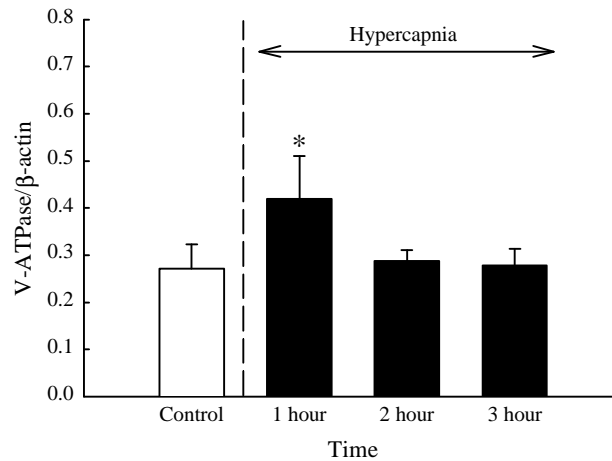


Figure 3. The temporal effects of external hypercapnia (N = 6 at each sampling time) on the levels of vacuolar H⁺V-ATPase B subunit mRNA in trout (*Oncorhynchus mykiss*) gill tissue. Transfers were hybridized with a homologous H⁺V-ATPase B subunit cDNA probe (810 base pairs) or with a homologous β-actin clone. Slot blots were loaded with 0.5 or 1.0 μg of total RNA obtained from perfused fish. * denotes a significant difference from the control value.

implants) for 4 days was associated with an approximate doubling of gill H⁺-ATPase mRNA levels (Figure 4). Thus, it is conceivable that the rise in cortisol levels during hypercapnia may act as a trigger for increased H⁺V-ATPase gene expression

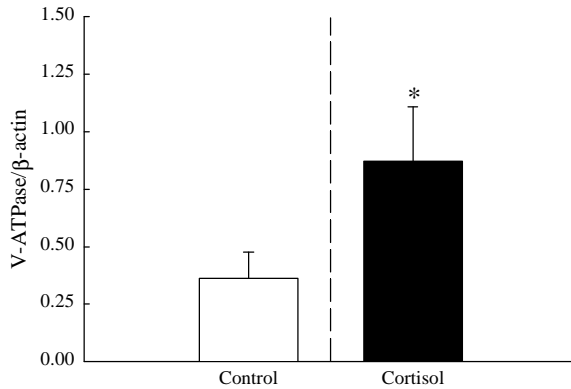
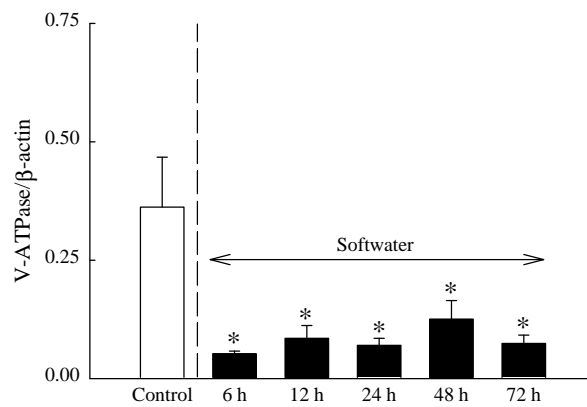


Figure 4. The effects of chronic cortisol elevation (N = 6) on the levels of vacuolar H⁺V-ATPase B subunit mRNA in trout (*Oncorhynchus mykiss*) gill tissue. Transfers were hybridized with a homologous H⁺V-ATPase B subunit cDNA probe (810 base pairs) or with a homologous β-actin clone. Slot blots were loaded with 0.5 or 1.0 μg of total RNA obtained from perfused fish. * denotes a significant difference from the control value.

Although a previous study has examined the impact of elevating the ionic composition of freshwater on branchial H⁺V-ATPase activity (Lin and Randall 1993), this is the first study to address the effects of reducing the ionic strength of water. Figure 5 illustrates that exposure of trout to ion-poor water was associated with a pronounced and sustained decrease in the levels of H⁺V-ATPase mRNA in gill tissue. Under normal conditions, there is an obligate linkage between H⁺ excretion and Na⁺ uptake. In the present study, the level of Na⁺ in the ambient water was lowered to values well below the K_m of the Na⁺ uptake mechanism and consequently Na⁺ uptake is depressed under such conditions (Perry and Laurent 1989). Thus, the decrease in gill H⁺V-ATPase

mRNA levels during exposure to ion-poor water may simply reflect a decreased need to excrete H^+ in the face of lowered Na^+ uptake rates. Interestingly, exposure of trout to ion-poor water is accompanied by proliferation of chloride cells on gill lamellae (Perry and Laurent 1989). Thus, the decrease in H^+V -ATPase mRNA levels at such times is further evidence that the pavement cell may be a more important site of H^+V -ATPase expression.



Acknowledgements

Figure 5. The temporal effects of exposure to ion poor water (N = 6 at each sampling time) on the levels of vacuolar H^+V -ATPase B subunit mRNA in trout (*Oncorhynchus mykiss*) gill tissue. Transfers were hybridized with a homologous H^+V -ATPase B subunit cDNA probe (810 base pairs) or with a homologous β -actin clone. Slot blots were loaded with 0.5 or 1.0 μ g of total RNA obtained from perfused fish. * denotes a significant difference from the control value.

This work was funded by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada to S.F.P and D.A.J. The authors would like to thank Dr. Pat Walsh for generously providing gill RNA from assorted fish species.

References

- Avella, M. and M. Bornancin. A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 142: 155-175 (1989).
- Chomczynski, P. and K. Mackey. Modification of the TRI Reagent™ procedure for the isolation of RNA from polysaccharide- and proteoglycan-rich sources. *Biotechniques* 19: 942-945 (1995).
- Chomczynski, P. and N. Sacchi. A single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochem.*: 156-159 (1987).
- Feinberg, A. P. and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochem.* 132: 6-13 (1983).
- Forgac, M. Structure, function and regulation of the vacuolar (H⁺)-ATPases. *FEBS Lett.* 440: 258-263 (1998).
- Goss, G. G., Perry, S. F., Fryer, J. N., and P. Laurent. Gill morphology and acid-base regulation in freshwater fishes. *Comp. Biochem. Physiol. A* 119: 107-115. 1998.
- Goss, G. G., Perry, S. F. and P. Laurent. 1995. Gill morphology and acid-base regulation. In *Fish Physiology Vol 14*. Edited by C. M. Wood and T. J. Shuttleworth. New York: Academic Press.
- Goss, G. G., Perry, S. F., Wood, C. M. and P. Laurent. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. *J. Exp. Zool.* 263: 143-159 (1992).
- Krogh, A. The active absorption of ions in some freshwater animals. *Z. Vergl. Physiol.* 25: 335-350 (1938).

- Kultz, D. and G. N. Somero. Osmotic and thermal effects on in situ ATPase activity in permeabilized gill epithelial cells of the fish *gillichthys mirabilis*. *J. Exp. Biol.* 198: 1883-1894 (1995).
- Laurent, P., Goss, G. G. and S. F. Perry. Proton pumps in fish gill pavement cells? *Arch. Int. Physiol. Biochim. Biop.* 102: 77-79 (1994).
- Lin, H., Pfeiffer, D. C., Vogl, A. W., Pan, J. and D. J. Randall. Immunolocalization of H⁺-ATPase in the gill epithelia of rainbow trout. *J. Exp. Biol.* 195: 169-183 (1994).
- Lin, H. and D. J. Randall. Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. Exp. Biol.* 161: 119-134 (1991).
- Lin, H. and D. J. Randall. H⁺-ATPase activity in crude homogenates of fish gill tissue - inhibitor sensitivity and environmental and hormonal regulation. *J. Exp. Biol.* 180: 163-174 (1993).
- Lin, H. and D. J. Randall. Proton Pumps in Fish Gills. In *Cellular and Molecular Approaches to Fish Ionic Regulation*. Vol. 14. Edited by C. M. Wood and T. J. Shuttleworth. New York: Academic Press (1995)
- Marshall, W. S. Transport Processes in Isolated Teleost Epithelia: Opercular Epithelium and Urinary Bladder. In *Cellular and Molecular Approaches to Fish Ionic Regulation*. Vol. 14. Edited by C. M. Wood and T. J. Shuttleworth. New York: Academic Press. (1995).
- Nelson, N. Evolution of organelar proton-ATPases. *Biochimica et Biophysica Acta* 1100: 109-124 (1992).
- Nelson, N. and W. R. Harvey. Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol. Rev.* 79: 361-385 (1999).
- Niederstaetter, H. and B. Pelster. Expression of two vacuolar-type ATPase B subunit isoforms in swimbladder gas gland cells of the European eel: nucleotide sequences and deduced amino acid sequences. *Biochim. Biophys. Acta* 1491: 133-142 (2000).

- Perry, S. F. The chloride cell: Structure and function in the gill of freshwater fishes. *Annu. Rev. Physiol.* 59: 325-347 (1997).
- Perry, S. F., M. L. Beyers and D. A. Johnson. Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H⁺-ATPase B sub-unit. *J. Exp. Biol.* 203: 459-470 (2000).
- Perry, S. F. and J. N. Fryer. Proton pumps in the fish gill and kidney. *Fish Physiol. Biochem.* 17: 363-369 (1997).
- Perry, S. F. and P. Laurent. Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the branchial chloride cell. *J. Exp. Biol.* 147: 147-168 (1989).
- Perry, S. F. and S. G. Reid. Injection techniques. In *Biochemistry and Molecular Biology of Fishes. Volume 3: Analytical Techniques*. Edited by P. W. Hochachka and T. P. Mommsen. Amsterdam: Elsevier. (1994).
- Sambrook, J. Fritsch, E. F. and T. Maniatis. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. (1989).
- Stevens, T. H. and M. Forgac. Structure, function and regulation of the vacuolar (H⁺)-ATPase. *Annu. Rev. Cell Dev. Biol.* 13: 779-808 (1997).
- Sullivan, G.V., Fryer, J. N. and S. F. Perry. Immunolocalization of proton pumps (H⁺-ATPase) in pavement cells of rainbow trout gill," *J. Exp. Biol.* 198: 2619-2629 (1995).
- Sullivan, G. V., Fryer, J. N. and S. F. Perry. Localization of mRNA for proton pump (H⁺-ATPase) and Cl⁻/HCO₃⁻ exchanger in rainbow trout gill. *Can. J. Zool.* 74: 2095-2103 (1996).
- Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J. and W. R. Harvey. Animal plasma membrane energization by proton-motive V-ATPases. *Bioessays* 8: 637-648 (1999).

**EFFECTS OF CHRONIC HYPERCAPNIA
ON THE EUROPEAN EEL (ANGUILLA ANGUILLA)**

D.J. McKenzie
School of Biosciences, University of Birmingham,
Birmingham, B15 2TT, U.K.
Tel. +39-0523-887939; Fax. +39-0523-889426;
E-mail: mckenzie@cram.enel.it

A.Z. Dalla Valle¹, M. Piccolella², G. Piraccini², E.W. Taylor¹,
C.L. Bolis² and J.F. Steffensen³

¹School of Biosciences, University of Birmingham;

²Institute of Pharmacological Sciences, University of Milan;

³Marine Biological Laboratory, University of Copenhagen.

EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Eels reared intensively in systems with recirculating water may experience severe chronic hypercapnia, with water CO₂ partial pressures (pwCO₂) exceeding 30 mmHg (Steffensen and Lomholt, 1990). This is over 10 times normal ambient pwCO₂ in surface waters (1 to 3 mmHg) and would cause heavy mortalities in cultured salmonids (Fivelstad et al., 1998).

Hypercapnia is stressful for fish because it acidifies the blood and impairs blood oxygen transport through Bohr and Root effects. Freshwater fish correct acid-base imbalances through dynamic manipulation of Na⁺ uptake in exchange for H⁺ (either with a Na⁺/H⁺ exchanger or via the action of a proton pump and Na⁺ channel) or Cl⁻ uptake in exchange for HCO₃⁻ (a Cl⁻/HCO₃⁻ exchanger), these processes occurring almost exclusively at the gills (see Heisler, 1993; Lin and Randall, 1996; Claiborne, 1998, for reviews). Internal acidosis, such as that elicited by hypercapnia, stimulates Na⁺/H⁺ exchange or inhibits Cl⁻/HCO₃⁻ exchange, causing excretion of acid equivalents to the water, a net accumulation of HCO₃⁻, and compensation of blood pH (Heisler, 1993; Claiborne, 1998).

Most freshwater teleosts rely on inhibition of $\text{Cl}^-/\text{HCO}_3^-$ exchange (Heisler, 1993; Claiborne, 1998). The eel is unusual in that it possesses very low or undetectable activity of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger and seems to rely on manipulation of Na^+ influx to correct acidosis and passive Cl^- loss to correct alkalosis (Bornancin et al., 1977; Hyde and Perry, 1987; 1989; Goss and Perry, 1994). It is reported to have a rather limited ability to compensate for acidosis, if compared with Rainbow trout (Hyde and Perry, 1987; 1989).

The acid-base consequences of acute and chronic exposure to severe hypercapnia in the eel were, therefore, investigated. In recirculating eel farms, it is probable that the eels actually experience chronic diurnal fluctuations in pwCO_2 that are linked to daily feeding schedules, rather than chronic exposure to a fixed degree of hypercapnia. Thus, the effects of chronic diurnal fluctuations in hypercapnia were also investigated.

Acute Hypercapnia

Acute exposure to progressive hypercapnia (20 min at pwCO_2 's of 10, 20, 40, 60 then 80 mmHg) caused a linear increase in arterial pCO_2 (paCO_2) from 3.5 ± 0.4 mmHg in normocapnia to 44.9 ± 2.6 at $\text{pwCO}_2 = 80$ mmHg. As can be seen in the Davenport (fig 1), the increase in paCO_2 was coupled to a severe decline in arterial pH (pHa), a decline that paralleled the non-bicarbonate buffer line for eel plasma (Hyde et al., 1987). The acidosis was linked to a profound (~ 80%) reduction in arterial total O_2 content (caO_2), presumably as a consequence of Bohr and Root effects. Nonetheless, all of the eels survived the acute hypercapnia protocol, and exhibited no significant changes in oxygen uptake or cardiac output.

Chronic Hypercapnia at Fixed Levels

Eels were reared for 6 months at pwCO_2 's of ambient (control), 15, 30 and 45 mmHg, and the effects on plasma acid base status measured. As can be seen in the Davenport diagram in fig 1, paCO_2 equilibrated at approximately 2 to 3 mmHg above pwCO_2 in the eels in each group, and this was associated with a progressive decline in pHa , which was significantly lower than the control eels in all hypercapnic groups. However, as is clear from fig 1, the eels accumulated significant amounts of HCO_3^- in the plasma, reaching over 70 mmol l^{-1} in the animals acclimated to $\text{pwCO}_2 = 45$ mmHg, such that pHa deviated significantly from the non-bicarbonate buffer line and, for any given paCO_2 , pHa was

significantly higher than under acute exposure conditions. This compensation of pHa was linked to a higher caO₂ for any given paCO₂ in the chronically versus the acutely exposed animals, such that only animals at pwCO₂ = 45 mmHg exhibited a significant hypoxaemia relative to the control eels.

The accumulation of bicarbonate is higher than has hitherto been considered possible for teleost fish; Heisler (1993) suggested that any accumulation of HCO₃⁻ above 30 mmol l⁻¹ would lead to too severe a reduction in plasma chloride levels. This because the accumulation of HCO₃⁻ is linked to the loss of a strong anion (Cl⁻) in order to protect electroneutrality. As can be seen in fig 2, there was an almost equimolar loss of Cl⁻ for every HCO₃⁻ accumulated by the eels exposed to chronic hypercapnia, such that the eels exposed to pwCO₂ = 45 mmHg had plasma Cl⁻ levels that were 50% of control animals, at around 75 mmol l⁻¹. This profound decline in plasma Cl⁻ was not linked to any significant changes in plasma Na⁺ levels or in plasma osmolarity. The ability of the eel to tolerate unusually low plasma Cl⁻ levels has been described previously (Farrell and Lutz, 1975).

Given the reported inability of the eel to manipulate Cl⁻/HCO₃⁻ exchange during extracellular acidosis (Bornancin et al., 1977; Hyde and Perry, 1987; 1989; Goss and Perry, 1994), the question arises as to how the eels in the current study were able to accumulate such large amounts of bicarbonate in the plasma. It has been suggested (Heisler, 1993) that the activity of a gill H⁺-ATPase alone could compensate an acidosis by removing protons and retaining bicarbonate, these two formed from the catalysed hydration of CO₂ in the gill epithelium. Indeed, Lin and Randall (1993) found that hypercapnia caused an increase in H⁺-ATPase activity in trout gills. However, chronic hypercapnia had no such effect on H⁺-ATPase activity in eel gills when assayed by the same technique, although it did cause an increase in Na⁺,K⁺-ATPase activity. Goss and Perry (1994) found evidence that the eel may regulate passive efflux of Na⁺ versus Cl⁻ to regulate strong ion difference. Thus, the hypercapnic eels may have let plasma Cl⁻ decline through loss to the water, and thereby accumulated HCO₃⁻ as a passive consequence of electroneutrality

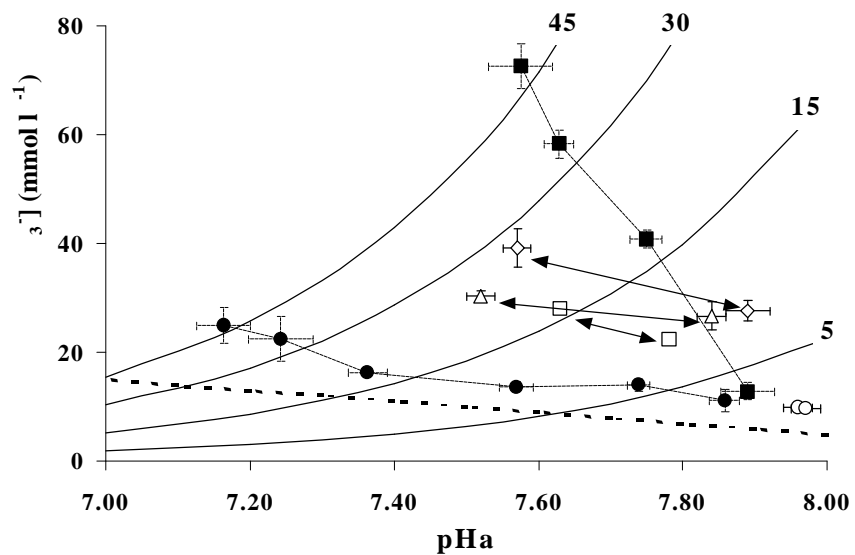


Figure 1. Davenport diagram showing the relationship between mean (\pm SE) arterial pH and bicarbonate concentration in eels exposed to acute hypercapnia (black circles), chronic fixed-level hypercapnia (black squares) and chronic diurnal fluctuations in hypercapnia (open symbols – squares = 5 to 15 mmHg pwCO₂; triangles = 5 to 25; diamonds = 5 to 35; circles = controls). See text for further details. n = between 4 and 7. Dotted line = non-bicarbonate buffer line for eel plasma (Hyde et al. 1987); curved solid lines are pCO₂ isopleths.

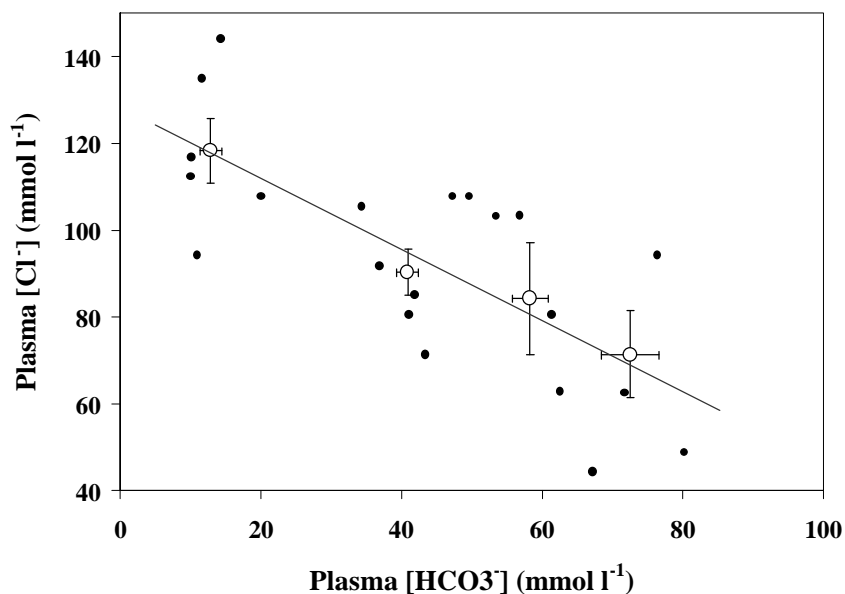


Figure 2. Relationship between plasma bicarbonate and chloride levels in eels exposed to chronic hypercapnia ($p\text{wCO}_2 = \text{ambient, 15; 30 or 45 mmHg}$ for 6 months). Black symbols are individual points; white symbols are mean (\pm SE) for each group ($n = 4$ to 6). Regression line describes linear relationship between individual data points, $y = -0.818x + 123$, $r^2 = 0.536$.

Despite the chronic extracellular acid-base disturbances, the eels regulated intracellular pH unchanged in muscle, liver and heart. As a consequence, calculation of tissue HCO_3^- levels indicated that they would not have risen above approximately 25 mmol l^{-1} , so that intracellular Cl^- levels would not have fallen to potentially dangerous levels (Heisler, 1993).

Chronic fluctuating hypercapnia

Eels were reared for 6 months with daily fluctuations in $p\text{wCO}_2$, of 5 to 15; 5 to 25, and 5 to 35, with the peak at 23:00 and the low at 11:00. Under these circumstances the animals exhibited diurnal fluctuations in acid-base status. As

can be seen from figure 1, the diurnal increase in pwCO_2 and paCO_2 was linked to a reduction in pHa , which paralleled the non-bicarbonate buffer line for eel plasma (Hyde et al., 1987). This indicates that under fluctuating conditions the animals were not able to accumulate bicarbonate as effectively as when exposed to fixed hypercapnic conditions. Both at the low and high point, the eels were acidotic relative to control animals maintained at ambient pwCO_2 . The acidosis was linked to chronic and quite severe hypoxaemia in all hypercapnic groups, relative to the control eels, although caO_2 did not fluctuate with the diurnal changes in pHa . It is a testament to the hardiness of the species that they were able to tolerate such severe diurnal acid-base disturbances and chronic hypoxaemia.

The diurnal fluctuations in pwCO_2 and plasma HCO_3^- were linked to fluctuations in plasma Cl^- levels, of up to 10 mmol^{-1} in the eels exposed to the 5 to 35 mmHg pwCO_2 regime (figure 3). This diurnal rise in Cl^- as paCO_2 and plasma HCO_3^- fell is difficult to explain given the reported absence of branchial $\text{HCO}_3^-/\text{Cl}^-$ exchange in the eel (Bornancin et al., 1977; Hyde and Perry, 1987; 1989; Goss and Perry, 1994).

Indicators of stress during chronic hypercapnia

In the eels exposed to chronic fixed hypercapnia, there were no indicators of stress such as elevated plasma cortisol or catecholamine levels, increased metabolic rate, or impaired exercise performance. This, however, was not true of the animals exposed to fluctuating hypercapnia. These exhibited a diurnal catecholamine release associated with the high point of the pwCO_2 cycle, a clear primary stress indicator. Although there was no effect of fluctuating hypercapnia on metabolic rate, exercise performance was compromised in all hypercapnic groups relative to the control eels, when measured at the low point of the fluctuating cycle. Thus, fluctuating hypercapnia is significantly more stressful to the eel than is fixed hypercapnia. Nonetheless, the absence of any differences in standard metabolic rate amongst the hypercapnic groups may indicate that acid-base regulation has a very low metabolic cost for the eel.

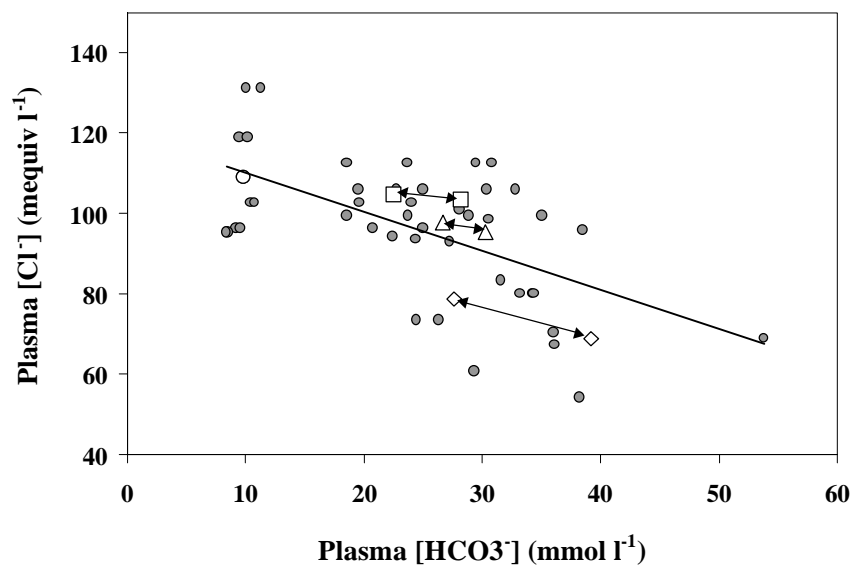


Figure 3. Relationship between plasma bicarbonate and chloride levels in eels exposed to chronic fluctuating hypercapnia ($p_w\text{CO}_2 = \text{ambient, 5 to 15; 5 to 25 and 5 to 35 mmHg}$, with peak at 23:00 and low at 11:00, for 6 months). Black symbols are individual points; white symbols are mean for each group ($n = 6$, squares = 5 to 15; triangles = 5 to 25; diamonds = 5 to 35 and circles = ambient). Regression line describes linear relationship between individual data points, $y = -0.971x + 120$, $r^2 = 0.340$.

Concluding Remarks

The results indicate that the eel is exceptionally tolerant of hypercapnia and the associated acidosis and hypoxaemia. The ability of the eel to tolerate such hypercapnic insults, including fluctuating hypercapnia, may be linked to their ability to survive (and exercise) in air when they migrate through wet vegetation in search of new habitats (Tesch, 1972; Berg and Steen, 1965; Hyde and Perry, 1987; Hyde et al., 1987). Hyde et al. (1987) showed that during air exposure the gills are no longer able to remove metabolic CO_2 and the animals exhibit a

mixed respiratory (i.e. hypercapnic) and metabolic acidosis. These authors noted the unusual tolerance of the animals for chronic hypoxaemia (Hyde et al., 1987). The evolution of the ability to survive what may be profound acid-base imbalances during air-exposure, particularly if associated with exercise, may, therefore, have pre-adapted the eel to the demanding conditions of intensive culture in recirculating systems.

Acknowledgements

This collaborative study was funded by the EU FAIR programme (Project CT96-1840). The authors are grateful to ENEL Ricerca Polo Ambiente for the use of the facilities at the La Casella Experimental Thermal Aquaculture Plant, where this research was conducted.

References

- Berg, T. and J.B. Steen. 1965. Physiological mechanisms for aerial respiration in the eel. *Comp. Biochem. Physiol.* 15: 469-484
- Bornancin, M.; G. De Renzis and J. Maetz. 1977. Branchial Cl transport, anion-stimulated ATPase and acid-base balance in *Anguilla anguilla* adapted to freshwater: Effects of hypoxia. *J. Comp. Physiol.* 117: 313-322.
- Claiborne, J.B. 1998. Acid-Base Regulation. In: *The Physiology of Fishes*, 2nd Ed. Edited by D.H. Evans, CRC Press, Boca Raton. pp. 343-378.
- Fivelstad, S., H. Haavik, G. Lovik and A.B. Olsen. 1998. Sublethal effects and safe levels of carbon dioxide in seawater for Atlantic salmon postsmolts (*Salmo salar* L.): Ion regulation and growth. *Aquaculture* 160: 305-316.
- Goss, G.G. and S.F. Perry. 1994. Different mechanisms of acid-base regulation in rainbow trout (*Oncorhynchus mykiss*) and American eel (*Anguilla rostrata*) during NaHCO₃ infusion. *Physiol. Zool.* 67: 381-406.
- Farrell, A.P. and P.L. Lutz. 1975. Apparent anion imbalance in the fresh water adapted eel. *J. Comp. Physiol.* 102: 159-166.

- Heisler, N. 1993. Acid Base Regulation. In: The Physiology of Fishes. Edited by D.H. Evans, CRC Press, Boca Raton. pp. 343-378.
- Hyde, D.A., T.W. Moon and S.F. Perry. 1987. Physiological consequences of prolonged aerial exposure in the American eel, *Anguilla rostrata*: blood respiratory and acid-base status. J. Comp. Physiol. 157B: 635-642.
- Hyde, D.A. and S.F. Perry. 1987. Acid-base and ionic regulation in the American eel (*Anguilla rostrata*) during and after prolonged aerial exposure: branchial and renal adjustments. J. Exp. Biol. 133: 429-447.
- Hyde, D.A. and S.F. Perry. 1989. Differential approaches to blood acid-base regulation during exposure to prolonged hypercapnia in two freshwater teleosts: The Rainbow trout (*Salmo gairdneri*) and the American eel (*Anguilla rostrata*). Physiol. Zool. 62: 1164-1186.
- Lin, H. and D.J. Randall. 1993. Proton-ATPase activity in crude homogenates of fish gill tissue: Inhibitor sensitivity and environmental and hormonal regulation. J. Exp. Biol. 180: 163-174.
- Lin H. and D.J. Randall. 1996. Proton pumps in fish gills. In: Fish Physiology Vol. XIV: Cellular and Molecular Approaches to Fish Ionic Regulation. Edited by C.M. Wood and T.S. Shuttleworth, Academic Press, San Diego. pp. 229-255.
- Steffensen, J.F. and J.P. Lomholt 1990. Accumulation of carbon dioxide in fish farms with recirculating water. In: Fish Physiology, Fish Toxicology and Fisheries Management. Edited by R.C. Ryan, EPA/600/9-90/011, Athens, Georgia. pp.157-161.
- Tesch, E. 1972. The Eel. Chapman and Hall, London. 276pp.

THE MUDSKIPPER *Periophthalmodon Schlosseri*
EXCRETES PROTONS TO LOWER EXTERNAL PH
AND TOXICITY OF AMMONIA

C. Barzaghi¹

¹Department of Biological Sciences, National University of Singapore,
10 Kent Ridge Road, Singapore 117543, Republic of Singapore.
Tel: (65) 874-2702. Fax: (65) 779-2486.

T. W. K. Kok¹, S. F. Chew², J. M. Wilson³, T. J. Lam¹,
D. J. Randall³, and Y. K. Ip¹

²Biology Division, Nanyang Technological University, National Institute of
Education, 469 Bukit Timah Road, Singapore 259756, Republic of Singapore,

³Zoology Department, University of British Columbia, Vancouver, Canada

EXTENDED ABSTRACT ONLY - DO NOT CITE

Many teleosts adopt the strategies of detoxifying ammonia to urea and/or glutamine during ammonia loading situations (Mommsen and Walsh, 1991). To date, only the mudskipper *Periophthalmodon schlosseri* is known to actively excrete ammonium ion in conditions of elevated ambient ammonia (Randall et al., 2000). Using artificial burrows made of rubber hose, we demonstrated that *P. schlosseri* was capable of sequestering at least 10 mM of ammonia to the external environment. Active pumping of NH_4^+ is energetically much more efficient than turning ammonia into urea or glutamine. One mole of ATP is utilized for every two moles of NH_4^+ eliminated. However, for such a mechanism to function, there must be means for the fish to prevent the back diffusion of NH_3 once the level of ammonia builds up in the environment. Therefore, it is logical to assume that this mudskipper would possess plasma membranes relatively impermeable to NH_3 and/or be capable of acidifying the external medium to keep NH_4^+ in the ionized form.

Our results verified that *P. schlosseri* was capable of manipulating the pH of the external medium. When placed in three and a half times its own volume of 50%

seawater at pH 8 and pH 9 in the presence of 2 mM Tris buffer, *P. schlosseri* could lower the external pH by 0.6 and 1.30 units, respectively, within 6 h. Stabilization of pH at a value around 7 occurred after approximately 12 h. This acidification occurred even if carbon dioxide excretion is removed by aeration.

Furthermore, *P. schlosseri* responded to the presence of NH_4Cl in the external medium by increasing the rate of excretion of proton equivalent. This unique capability of *P. schlosseri* to excrete proton equivalents in response to the presence of ammonia in the external medium would maintain the actively excreted NH_4^+ in the ionized form and prevent it from diffusing back to the tissues as NH_3 .

In its natural habitat, acidification of a small amount of burrow water by *P. schlosseri* is feasible since the burrow is poorly flushed. Mudskippers are the only fish known to rear the developing embryos in their burrows. The growth of embryos requires the mobilization of yolk and results in ammonia production and accumulation in the burrow water. Acidification of the burrow water lowers the concentration of NH_3 in the external medium and therefore reduces the toxicity of ammonia to both the embryos and the fish themselves.

References

- Mommsen, T. P. and P. J. Walsh. 1991. Urea synthesis in fishes: Evolutionary and Biochemical Perspectives. In "Biochemistry and molecular biology of fishes, 1. Phylogenetic and biochemical perspectives". (P. W. Hochachka and T. P. Mommsen, eds). pp 137-163. Elsevier.
- Randall, D. J., J. M. Wilson, K. W. Peng, T. W. K. Kok, S. S. L. Kuah, S. F. Chew, T. J. Lam and Y. K. K. Ip. 2000. The mudskipper *Periophthalmodon schlosseri* actively transports NH_4^+ against a concentration gradient and survives in dilute sea-water containing 100 mM NH_4Cl . *Am. J. of Physiol.* 277: R1562-R1567.

AMMONIA EXCRETION IN THE MUDSKIPPER,

PERIOPHTHALMODON SCHLOSSERI.

D.J.Randall¹,
Tel: 604-822-5709; Fax: 604-822-2416;
e-mail: Randall@zoology.ubc.ca

J. Wilson¹, C. Barzagli³, S.F. Chew² and Y.K. Ip³

- 1). Zoology, UBC; Vancouver, BC, Canada
2). Biology Division, Faculty of Science, Nanyang Technological University, Singapore, and 3). School Biol. Sci. National Univ. Singapore.

EXTENDED ABSTRACT ONLY – DO NOT CITE

The mudskipper, *Periophthalmodon schlosseri*, can survive air exposure for seven days, but drowns if denied access to air. This fish holds large volumes of air in its buccal cavity and exchanges gases across the buccal and opercular epithelium between blood and air. The gills are not involved in gas transfer (Wilson et al., 1999;) but are involved in ammonium ion transport. *Periophthalmodon schlosseri* can tolerate ammonia concentrations of over 100mM NH₄Cl in an external environment of 50% seawater (Peng et al., 1998). It appears that *P. schlosseri* is able to tolerate high environmental ammonia concentrations by actively excreting ammonium ions across the gills, thereby maintaining low tissue ammonia concentrations (Randall et al., 1999). Immunohistological studies (Wilson et al., 2000) have resulted in the localization of a number of transport proteins in the mitochondrial rich cells of the mudskipper gills (Figure 1). The possible role of these transport proteins in active ammonia excretion across the *Periophthalmodon schlosseri* gill will be discussed.

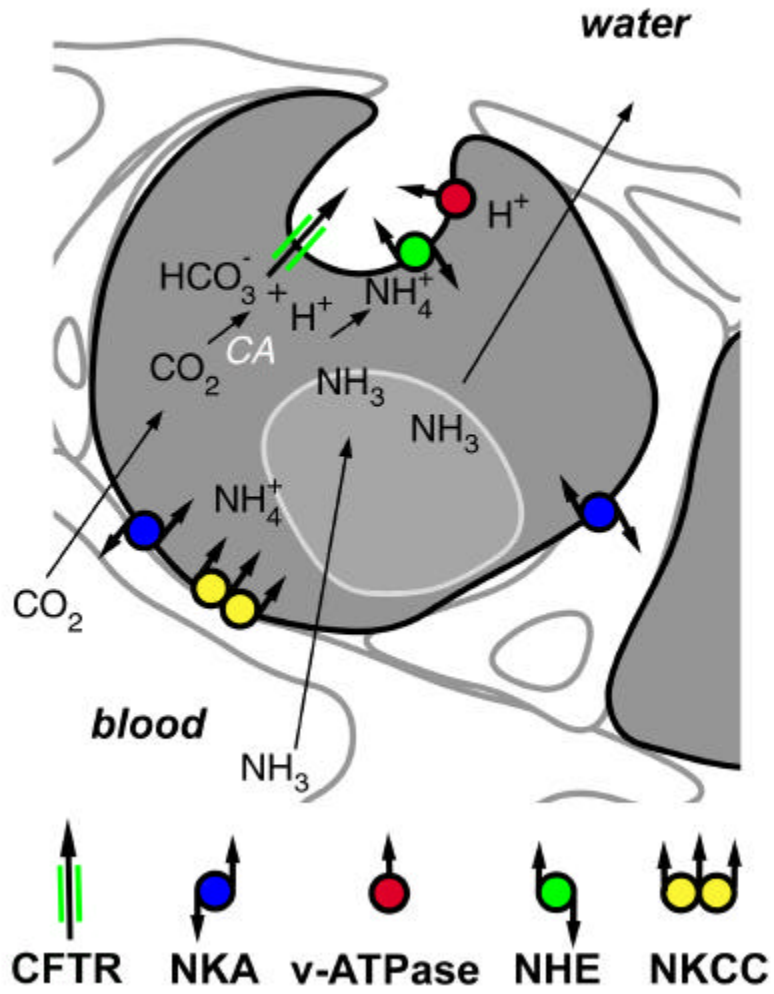
References

- Peng, K.W; S.F. Chew; C.B. Lim; T.W.K.Kok; S.S.L.Kuah; and Y.K. Ip. 1998. The mudskippers *Periophthalmus schlosseri* and *Boleophthalmus boddarti* can tolerate 446µM and 36 µM of environmental NH₃, respectively. Fish Physiol. Biochem. 19: 59-69.

- Wilson, J.M., Randall, D.J., Donowitz, M., Vogl, W. and Ip, A.K.Y., 2000. Immunolocalization of ion transport proteins to the mudskipper (*Periophthalmodon schlosseri*) branchial epithelium mitochondria-rich cells. *J. Exp. Biol.* In press.
- Randall, D.J., J.M. Wilson, K.W. Peng, T.W.K. Kok, S.S.L. Kuah, S.F. Chew, T.J. Lam and Y.K.Ip, 1999. The mudskipper, *Periophthalmodon schlosseri*, actively transports NH_4^+ against a concentration gradient. *Am. J. Physiol* 277 (Regulatory and Integrative Comp. Physiol.) 46: R1562-R1567
- Wilson, J.M., T.W.K. Kok, D.J.Randall, W.A.Vogl, and Y.K.Ip, 1999 The fine structure of the gill of the terrestrial mudskipper, *Periophthalmodon schlosseri*. *Cell & Tissue Res* 298: 345-356

Figure 1. Schematic of the mitochondrial rich cell of the gills of the mudskipper, *Periophthalmodon schlosseri*, showing the probable location of various transport proteins. CFTR, anion channel; NKA, sodium/potassium ATPase; v-ATPase, proton ATPase; NHE, sodium/proton exchange; NKCC, sodium/potassium/2chloride cotransport.

Mudskipper Model



**HCO₃⁻ DEHYDRATION IN THE PLASMA OF RAINBOW TROUT:
THE ROLE OF BUFFERING CAPACITY**

K.M. Gilmour

Department of Biology, Carleton University, 1125 Colonel By Dr.,
Ottawa, ON, K1S 5B6, Canada
Phone (613) 520-2600 x1608; Fax (613) 520-2569;
E-mail kgilmour@ccs.carleton.ca

P.R. Desforges

Department of Biology, University of Ottawa

Abstract

HCO₃⁻ entry into the red blood cell is thought to be the rate-limiting step in CO₂ excretion in fish. By theory, the addition of bovine carbonic anhydrase (CA) to the circulation should provide an extracellular site for HCO₃⁻ dehydration, enhancing CO₂ excretion, and lowering the post-branchial blood CO₂ tension (PaCO₂). In agreement with theory, intravascular injection of bovine CA (5 mg kg⁻¹) into rainbow trout caused a small but significant reduction in PaCO₂. The contribution of extracellular HCO₃⁻ dehydration to CO₂ excretion may, however, be limited by proton availability owing to the low nonbicarbonate buffering capacity (β) of trout plasma. To test this hypothesis, the effect on the plasma HCO₃⁻ dehydration rate of manipulating plasma β using HEPES was examined both *in vivo* and *in vitro*. *In vivo*, a greater than three-fold increase in plasma β (from -3.8 to -14 mmol L⁻¹ pH unit⁻¹) had no significant impact on the extent of the PaCO₂ decrease following CA injection. However, an increase in plasma β from -4.9 to -12 mmol L⁻¹ pH unit⁻¹ increased the *in vitro* HCO₃⁻ dehydration rate of separated plasma significantly in both the absence and presence of bovine CA. These data suggest that HCO₃⁻ dehydration in fish plasma is limited by proton availability. Under normal conditions *in vivo*, however, treatment with HEPES to increase plasma buffering capacity has little effect on the decrease in PaCO₂ achieved by the addition of bovine CA to the circulation of the fish because proton availability for HCO₃⁻ dehydration is so much greater in the red cell owing to both the buffering power of haemoglobin, and the oxylabile release of protons from haemoglobin (Haldane effect).

Introduction

CO₂ excretion in rainbow trout (*Oncorhynchus mykiss*) follows the typical vertebrate pattern, in which the majority of CO₂ is carried as HCO₃⁻ ions in the blood plasma (reviewed by Perry, 1986; Tufts and Perry, 1998). At the gills, HCO₃⁻ ions enter the red blood cell (RBC) via a Cl⁻/HCO₃⁻ exchanger (AE1 or band 3 protein) and are dehydrated to molecular CO₂, which then diffuses out of the blood to the ventilatory water along its partial pressure gradient. The protons required for HCO₃⁻ dehydration are provided primarily by haemoglobin, either through its buffering capacity or the Haldane effect, and the dehydration reaction is catalysed by RBC carbonic anhydrase (CA). The rate-limiting step in this process is thought to be HCO₃⁻ entry in the RBC via the anion exchanger (Perry and Gilmour, 1993, reviewed by Tufts and Perry, 1998). Because plasma CO₂ reactions in rainbow trout do not have access to extracellular CA activity at the gills (Perry et al., 1997; Gilmour et al., 1999), all catalysed HCO₃⁻ dehydration for CO₂ excretion must take place through the RBC. HCO₃⁻ dehydration in the plasma occurs at the uncatalysed rate and therefore makes a negligible contribution to CO₂ excretion owing to the brief residence time of blood in the gill [\sim 1-3 sec (Cameron and Polhemus, 1974) versus \sim 25-90 sec for the half-time of the uncatalysed HCO₃⁻ dehydration reaction at fish body temperatures (Perry, 1986)].

Given that CO₂ excretion is limited by HCO₃⁻ entry into the RBC, the addition of CA to the blood plasma of rainbow trout would be predicted to enhance CO₂ excretion by providing an extracellular site for HCO₃⁻ dehydration. However, experimental results to date do not appear to support this prediction. Wood and Munger (1994) found that the effects of CA injection (10 mg kg⁻¹) injection into resting rainbow trout were similar to those of saline injection - very minor decreases in arterial CO₂ tension (PaCO₂) and HCO₃⁻ concentration and increases in arterial pH (pHa) were observed, changes that were close to the limit of reliable detection. Similar results (small increases in pHa, little or no change in PaCO₂ or blood total CO₂ content) were obtained by Lessard et al. (1995) and Gilmour et al. (1994).

The apparent lack of significant effect of exogenous CA injection on CO₂ excretion in trout may be the consequence of limited proton availability in plasma. The nonbicarbonate buffering capacity (β) of separated trout plasma is -2.6 mmol L⁻¹ pH unit⁻¹, whereas that of whole blood (at a haematocrit of 25%) is -9.7 mmol L⁻¹ pH unit⁻¹ (Wood et al., 1982), the higher value for whole blood

reflecting the buffering properties of haemoglobin. Because the dehydration of one mole of HCO_3^- consumes one mole of H^+ , HCO_3^- dehydration in the plasma of rainbow trout may be limited by proton availability owing to the relatively low plasma buffering capacity. Thus, the objective of the present study was to test the hypothesis that an increase in plasma nonbicarbonate buffering capacity would increase the extent to which exogenous CA activity enhanced (plasma) HCO_3^- dehydration in rainbow trout both *in vivo* and *in vitro*.

Materials and Methods

Experimental animals.

Rainbow trout were obtained from Linwood Acres Trout Farm (Campbellcroft, ON) and maintained on a 12L:12D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechlorinated City of Ottawa tap water at 13°C. Trout were fed to satiation on alternate days with commercial trout pellets and food was withheld for 24 h prior to experimentation. Two groups of fish were used; one group ($N = 13$) acted as blood donors for *in vitro* experiments while blood respiratory variables were measured *in vivo* using an extracorporeal arterial blood loop on the second group of fish [mass = 527 ± 22 (mean \pm SEM); $N = 48$].

Surgical procedures.

All fish were anaesthetised in an aerated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g L^{-1}) and then transferred to an operating table that permitted the gills to be irrigated with the same anaesthetic solution throughout the surgery. For the fish used as blood donors for *in vitro* experiments, a single indwelling polyethylene cannula (Clay-Adams PE 50) was inserted into the dorsal aorta according to the basic method of Soivio et al. (1975). For continuous measurements of blood respiratory variables *in vivo* using the extracorporeal loop, the caudal vein and caudal artery were cannulated. Cannulation of the caudal vessels involved making a lateral incision at the level of the caudal peduncle so as to separate the epaxial and hypaxial musculature and expose the haemal arch. Catheters (PE50) were then fed into the caudal artery and vein in the anterior direction and were secured with ligatures to the skin; the incision was tightly closed with silk sutures. Small (1 cm^2) brass plates were stitched to the external surface of each operculum to allow the measurement of ventilation parameters using an impedance converter. After

surgery, fish were transferred to individual opaque acrylic boxes supplied with aerated, flowing water for a 24 h recovery period. Cannulae were flushed with heparinised (100 i.u. mL⁻¹ sodium heparin) Cortland saline.

Experimental protocol – In vitro experiments.

The objective of this series of experiments was to assess HCO₃⁻ dehydration rates *in vitro* in separated plasma under various conditions of β, CA activity and plasma HCO₃⁻ concentrations ([HCO₃⁻]). HCO₃⁻ dehydration rates were measured using the radioisotopic assay of Wood and Perry (1991). Approximately 30 mL of separated plasma was required for a typical single experimental run (*i.e.* *N* = 1). Thus, it was necessary to use pooled blood obtained by slow withdrawal from the dorsal aortic cannulae of 2-3 fish. Following blood withdrawal/pooling and separation by brief centrifugation to obtain plasma, 1.2 mL of 180 mmol L⁻¹ HEPES was added to one half of the plasma pool to achieve a nominal final concentration of 10 mmol L⁻¹ HEPES; β was measured for both the control (*i.e.* naturally-occurring β) and HEPES-treated plasma (see below). For each β, three levels of CA activity (0, 0.001 and 1 mg mL⁻¹) and two levels of HCO₃⁻ (nominally 10 and 15 mmol L⁻¹) were tested. Bovine CA was added (50 μl injection volume, bovine CA dissolved in saline) immediately prior to assaying the sample. Plasma HCO₃⁻ levels were elevated abruptly at the start of the assay by ‘spiking’ the plasma with 10 or 20 μL of isotope prepared in 500 mmol L⁻¹ NaHCO₃. The nominal HCO₃⁻ levels were verified by analysing the plasma total CO₂ concentration (CCO₂); actual HCO₃⁻ concentrations were 11.0 ± 0.3 (48) and 15.5 ± 0.3 (48) mmol L⁻¹ [mean ± SEM (*N*)]. Plasma pH was measured prior to and following the assay (pH glass and reference electrodes in a thermostatted blood gas cell connected to a blood gas analyser; Cameron Instruments).

The radioisotopic HCO₃⁻ dehydration assay was carried out as described by Wood and Perry (1991). In brief, plasma samples (0.8 mL) were equilibrated with a humidified gas mixture of 0.5% CO₂ in air (GF-3/MP gas mixing flowmeter; Cameron Instruments) for 60 min in a shaking water bath held at 10°C. To start the assay, 74 kBq of sodium [¹⁴C]bicarbonate was added to each sample and the vial containing the sample was then immediately sealed with a cap containing a CO₂ trap (a filter paper impregnated with 150 μL hyamine hydroxide). At the end of the 3 min assay period, filter paper and plasma ¹⁴C activities were determined by liquid scintillation counting (Packard TR 2500) with automatic quench correction. Filter papers were counted in 10 mL of Bio-

Safe NA (Research Products Int.) while 50 μL of plasma was counted in 10 mL of ACS (Amersham) scintillation cocktail. Plasma CCO_2 was measured on 20 μL duplicate samples (Capni-Con 5 total CO_2 analyser; Cameron Instruments). The HCO_3^- dehydration rate for each vial was then calculated by dividing filter paper ^{14}C activity by plasma specific activity and time.

Experimental protocol – In vivo experiments.

The objective of this series of experiments was to assess the effects of exogenous CA injection on CO_2 excretion *in vivo* under normal conditions and following elevation of plasma β by treatment with HEPES. CO_2 excretion was assessed indirectly by monitoring arterial CO_2 tension (PaCO_2) and pH (pHa) for 90 min following the intra-arterial injection of either 5 mg kg^{-1} bovine CA (in 1 mL saline) or the saline vehicle only. The effects of these treatments in fish at their naturally-occurring plasma β were compared with those in fish that had received an injection of HEPES (2 mL kg^{-1} of 1.5 mol L^{-1} stock solution to achieve a nominal final circulation concentration of 10 mmol L^{-1} HEPES) to elevate plasma β 2 h prior to CA or saline injection; plasma β was measured at the end of each experiment.

Blood respiratory variables were measured using an extracorporeal shunt in which blood was withdrawn from the caudal artery cannula and passed by means of a peristaltic pump through an external circuit containing pH, PCO_2 and PO_2 electrodes (Thomas, 1994). The flow rate through the external loop, which contained approximately 1 mL of blood, was 0.40 mL min^{-1} . To prevent clotting, the circuit was rinsed with at least 10 mL of heparinised (540 i.u. mL^{-1}) saline before initiating the blood flow. Arterial blood pH, PCO_2 and PO_2 were monitored using Radiometer or Cameron Instruments (CO_2 , O_2), and Metrohm (pH) electrodes housed in thermostatted cuvettes and connected to a blood gas analyser (Cameron Instruments). Blood gas electrodes were calibrated by pumping water equilibrated with appropriate gas mixtures (supplied by a GF-3/MP gas mixing flowmeter; Cameron Instruments) through the circuit; precision buffer solutions were used to calibrate the pH electrode. In addition to the blood respiratory variables, the frequency and amplitude of opercular displacements were assessed as an index of ventilation using a custom-built impedance converter that measured the changes in impedance between the electrodes sutured to the opercula. All analog signals (blood gases and impedance) were converted to digital data by interfacing with a data acquisition system (Biopac Systems Inc.) using Acknowledge™ data acquisition software

and a Pentium™ PC. Thus, continuous data recording were obtained for blood gases (PaO₂, PaCO₂) and pH (pHa) as well as ventilation frequency (V_f; automatic rate calculation from the raw impedance trace) and amplitude (V_{amp}, the difference between maximum and minimum impedance values).

Measurement of plasma nonbicarbonate buffering capacity.

To measure plasma β in either series of experiments, a plasma sample of approximately 3 mL was gassed first with CO₂ for 5 min and then several times briefly with air. Following each equilibration, approximately 0.7 mL of plasma were withdrawn and analysed for CCO₂ (20 μL in duplicate; Capni-Con 5 total CO₂ analyser; Cameron Instruments) and pH (pH glass and reference electrodes in a thermostated blood gas cell connected to a blood gas analyser; Cameron Instruments). Total CO₂ was plotted against pH and the nonbicarbonate buffering capacity was taken as the slope of the resultant linear regression line.

Statistical analysis.

Data are presented as means ± 1 standard error of the mean (SEM) (*N*). For *in vivo* experiments, mean blood gas and ventilatory data were compiled for 2 min periods at time = 0, immediately prior to saline or CA injection, and time = 90 min after saline or CA injection. Differences between the time = 0 and time = 90 min samples within a treatment were analysed statistically using a paired Student's *t*-test. A two-way repeated measures ANOVA followed by Tukey's post-hoc multiple comparisons test (as appropriate) was used to analyse the *in vitro* data for statistically-significant differences. In all cases, the fiducial limit of significance was 5%.

Results and Discussion

The addition of bovine CA to the circulation of rainbow trout resulted in a small, but significant, decrease in PaCO₂ coupled with a corresponding (non-significant) increase in pHa, whereas saline injection was without effect on either variable (Table 1). However, while HEPES treatment was successful in elevating the nonbicarbonate buffer capacity of the plasma more than three-fold (a significant increase from -3.82 ± 0.38 to -14.0 ± 2.76 mmol L⁻¹ pH unit⁻¹; unpaired Student's *t*-test, *P* < 0.05), this increase in plasma β did not enhance the effect of CA injection *in vivo*. As in fish of normal plasma β, CA injection in HEPES-treated trout was associated with a small, significant decrease in

PaCO₂ of about 0.2 Torr (Table 1). Again, saline injection was without effect on either PaCO₂ or pHa (Table 1), and ventilation in all groups was unaffected by either saline or CA injection (data not shown).

Table 1. The effect of saline or bovine CA (5 mg kg⁻¹) injection on arterial PCO₂ and pH in control fish and fish treated with HEPES to elevated plasma nonbicarbonate buffer capacity.

Treatment	pHa		PaCO ₂ (Torr)	
	Time = 0 min	Time = 90 min	Time = 0 min	Time = 90 min
Control β, saline (12)	7.92 ± 0.02	7.92 ± 0.02	2.01 ± 0.15	1.95 ± 0.14
Control β, CA (22)	7.90 ± 0.02	7.93 ± 0.03	2.54 ± 0.17	2.30 ± 0.16*
High β, saline (6)	7.80 ± 0.05	7.83 ± 0.04	2.22 ± 0.19	2.30 ± 0.17
High β, CA (8)	7.94 ± 0.03	7.95 ± 0.04	1.73 ± 0.16	1.54 ± 0.15*

* indicates a significant difference (paired *t*-test, *P* < 0.05) within a treatment from the corresponding time = 0 value.

On the basis of these data, it is clear that the addition of exogenous CA to the circulation of rainbow trout enhances CO₂ excretion, presumably by providing an extracellular site for HCO₃⁻ dehydration. However, the extent to which exogenous CA enhanced CO₂ excretion was limited, nor was this limitation relieved by increasing the plasma nonbicarbonate buffering capacity. There are at least two plausible explanations for these results: (1) CO₂ excretion *in vivo* under resting conditions is not limited to a significant extent by the rate of anion exchange across the RBC, and hence bypassing this step through the addition of extracellular CA to the circulation has little or no effect on PaCO₂; or (2) HCO₃⁻ dehydration in the plasma is limited by proton availability, even under conditions of elevated plasma β, or is limited by some factor other than proton availability.

The possibility that CO₂ excretion *in vivo* under resting conditions is not limited by access of plasma HCO₃⁻ to red cell CA via Cl⁻/HCO₃⁻ exchange was also raised by Wood and Munger (1994) to explain the negligible effect of CA injection on resting PaCO₂ in their experiments. By contrast, the large, significant elevation of PaCO₂ that normally follows exhaustive exercise was attenuated by 50% in rainbow trout treated with CA prior to exercise, suggesting that the rate of anion exchange is limiting under conditions of elevated CO₂ production (Wood and Munger, 1994). Similarly, Lessard et al. (1995) found

little effect of CA injection in rainbow trout under normoxic conditions but calculated that PaCO₂ would be decreased significantly (by ~1 Torr) by CA infusion during hypoxia, suggesting that the rate of anion exchange would be limiting under conditions of elevated CO₂ excretion. The hypothesis that CO₂ excretion *in vivo* is limited by entry of plasma HCO₃⁻ into the red cell only under conditions of elevated CO₂ production or excretion is attractive in terms of explaining the small effect of CA injection on PaCO₂ under resting, normoxic conditions observed in the present study and the lack of effect observed previously (Wood and Munger, 1994; Lessard et al., 1995). However, this hypothesis also necessitates that some other factor in the CO₂ excretion pathway acts to limit CO₂ excretion under normoxic conditions at rest, and it is difficult to conceive of what this factor would be. CO₂ diffusion in biological media is rapid and should never be rate-limiting during the course of CO₂ transfer (Swenson, 1990), yet CO₂ excretion *in vivo* is thought to behave as a diffusion-limited system (Malte and Weber, 1985; Julio et al., 2000). Recently, the apparent diffusion limitations on CO₂ excretion in fish were demonstrated experimentally to be the consequence of chemical reaction limitations (Julio et al., 2000). Julio et al. (2000) found that the increase in PaCO₂ elicited by reducing the surface area for gas exchange using gill ligation in rainbow trout could be reversed by treatment of the fish with bovine CA. The demonstration that CO₂ excretion *in vivo* is limited by chemical reaction kinetics, coupled with the fact that CA injection into normoxic, resting rainbow trout does a produce significant decrease in PaCO₂ (Table 1), confirm that HCO₃⁻ entry to the RBC via the anion exchanger is indeed the rate limiting step in CO₂ excretion *in vivo*.

Given Cl⁻/HCO₃⁻ exchange as the rate-limiting step in CO₂ excretion *in vivo*, it might be expected that bypassing this step by providing a site for extracellular HCO₃⁻ dehydration at the catalysed rate would result in larger decreases in PaCO₂ than have been measured experimentally (Table 1; Wood and Munger, 1994; Lessard et al., 1995). The small effect of CA injection on PaCO₂ *in vivo* suggests that HCO₃⁻ dehydration in the plasma is limited by some factor, and an obvious candidate for such a factor is the plasma nonbicarbonate buffering capacity, since each mole of HCO₃⁻ ions dehydrated requires a mole of protons. Typically, the arterial-venous difference in blood total CO₂ concentration in rainbow trout is about 1.5 mmol L⁻¹ (Brauner, 1995). This clearance of CO₂ as the blood passes through the gills is accompanied by a decrease in PCO₂ from about 3.7 Torr in the pre-branchial blood to about 2.3 Torr in the post-branchial blood (Tufts and Perry, 1998). The arterial-venous pH difference is dependent upon a variety of environmental factors (Brauner and Randall, 1998), but tends

to be small under resting, normoxic conditions (arterial pH > venous pH) (Brauner, 1995). Thus, under normal conditions, $\sim 1.5 \text{ mmol L}^{-1} \text{ HCO}_3^-$ is lost from the plasma with little change in pH (Fig. 1); the $1.5 \text{ mmol L}^{-1} \text{ H}^+$ required for HCO_3^- dehydration is provided by the buffering capacity of haemoglobin and by Bohr protons released from haemoglobin during oxygenation (Tufts and Perry, 1998; Brauner and Randall, 1998). However, were HCO_3^- dehydration to take place only in the plasma, where β is $-2.6 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$ (rather than the whole blood value of $-9.7 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$), a decrease in PCO_2 from 3.7 to 2.3 Torr would elicit only an approximately 0.5 mmol L^{-1} decrease in plasma HCO_3^- ; a 1.5 mmol L^{-1} change in the plasma HCO_3^- concentration would require an approximately 6 Torr decrease in PCO_2 together with a 0.5 unit pH change (Fig. 1). Clearly, by theory the low buffering capacity of the plasma will limit proton availability and hence constrain the extent of HCO_3^- dehydration that can take place in this compartment.

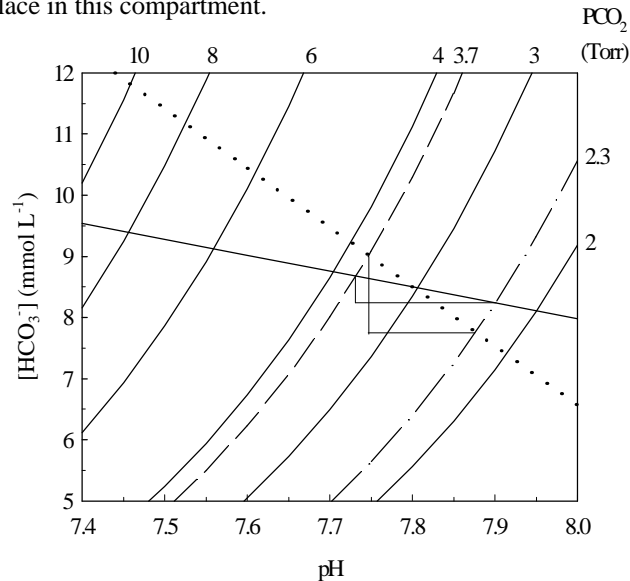


Figure 1. A pH- HCO_3^- diagram for rainbow trout at 13°C . The PCO_2 for a given combination of pH and $[\text{HCO}_3^-]$ was calculated using the Henderson-Hasselbalch equation and the appropriate values for pK' and αCO_2 (Boutilier et al., 1984). Venous (dashed line) and arterial (dot-dash line) PCO_2 values for rainbow trout were drawn from Tufts and Perry (1998). Buffer lines for rainbow trout whole blood (dotted line) and separated plasma (solid line) were constructed using buffer values, β , derived by Wood et al. (1982). $\Delta[\text{HCO}_3^-]$ and ΔpH for the arterial-venous PCO_2 decrease at each β are drawn.

The results of the *in vitro* experiment support the hypothesis that proton availability limits HCO_3^- dehydration in rainbow trout plasma. HCO_3^- dehydration rates of separated plasma at the natural buffering capacity of the fish or at elevated buffering capacity (HEPES-treated plasma) were measured at three levels of CA activity and two HCO_3^- concentrations using the radioisotopic assay of Wood and Perry (1991). As expected on the basis of previous studies (e.g. Wood and Perry, 1991; Perry and Gilmour, 1993), the HCO_3^- dehydration rate for separated plasma was low in the absence of added CA activity and increased in a dose-dependent fashion with added bovine CA (Fig. 2). Manipulation of the plasma HCO_3^- concentration had little effect on plasma HCO_3^- dehydration rates at any CA level (Fig. 2), a finding similar to that of Perry and Gilmour (1993). However, addition of 10 mmol L^{-1} HEPES to separated plasma to elevate the nonbicarbonate buffer capacity from -4.9 ± 0.7 to $-12.1 \pm 1.4 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$, a significant (paired Student's *t*-test, $P < 0.05$) approximately 2.5-fold rise, increased the HCO_3^- dehydration rate significantly at all CA levels (Fig. 2). Moreover, the higher HCO_3^- dehydration rates in HEPES-treated plasma samples were achieved with plasma pH increases that were generally smaller, although not significantly so, than those in plasma samples at the naturally-occurring buffer capacity (Table 2), despite the presumably greater consumption of protons to support the higher HCO_3^- dehydration rates.

Table 2. Mean changes in plasma pH ($\Delta\text{pH} = \text{final pH} - \text{initial pH}$) as a result of the *in vitro* HCO_3^- dehydration assay.

[CA] (mg mL^{-1})	Low β , low [HCO_3^-]	Low β , high [HCO_3^-]	High β , low [HCO_3^-]	High β , high [HCO_3^-]
0 ($N = 6$)	0.05 ± 0.19	0.23 ± 0.24	0.17 ± 0.14	0.20 ± 0.13
0.001 ($N = 6$)	0.45 ± 0.19	0.41 ± 0.23	0.34 ± 0.13	0.34 ± 0.13
1 ($N = 6$)	0.53 ± 0.19	0.39 ± 0.24	0.34 ± 0.15	0.40 ± 0.17

No significant differences in ΔpH were observed within a treatment.

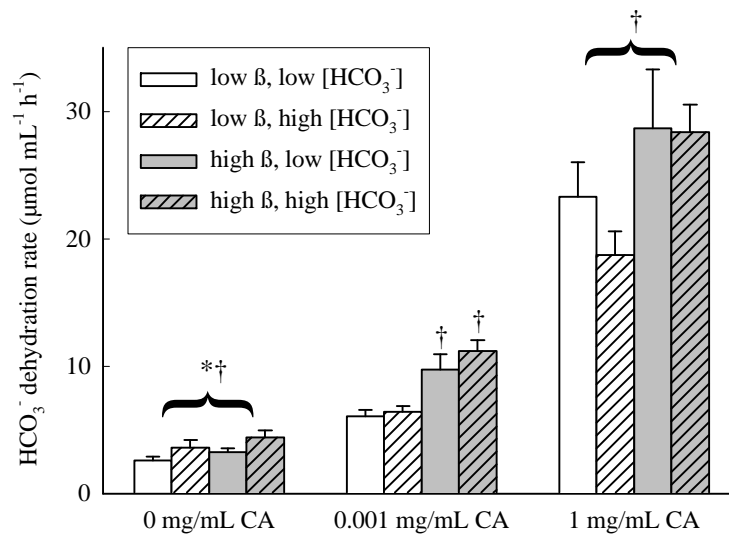


Figure 2. Mean HCO₃⁻ dehydration rates ($N = 6$ for all groups) for separated plasma samples from rainbow trout under various conditions of nonbicarbonate buffer capacity (β), CA activity and plasma HCO₃⁻ concentration ([HCO₃⁻]). * indicates a significant effect of [HCO₃⁻] while † indicates a significant effect of β on the HCO₃⁻ dehydration rate (two-way repeated measures ANOVA followed by Tukey's all-pairwise post-hoc multiple comparisons test at each CA level, $P < 0.05$). Use of the bracket denotes that no significant interaction occurred between the two factors, [HCO₃⁻] and β , such that multiple comparisons were carried out only within a factor and not among all four groups in a treatment.

Thus, at least *in vitro*, proton availability appears to be a limiting factor on HCO₃⁻ dehydration in rainbow trout plasma. Interestingly, limitations on proton availability owing to the relatively low buffering capacity of plasma are thought to restrict the contribution of pulmonary capillary endothelial CA (CA IV) in mammals to less than 10% of CO₂ excretion (Bidani and Heming, 1991; Bidani,

1991; Heming and Bidani, 1992). By contrast, the high buffering capacity of dogfish plasma relative to the whole blood value may allow gill membrane-bound CA IV in this species to make a significant (30% or more) contribution to CO₂ excretion (Gilmour et al., 1999). In rainbow trout, however, even when the plasma nonbicarbonate buffer capacity was increased more than three-fold, the impact of exogenous CA activity on PaCO₂ was small (Table 1). This result suggests that, under normal conditions, the high availability of protons for HCO₃⁻ dehydration in the red cell is of critical importance to CO₂ excretion. That is, in the presence of extracellular CA and elevated plasma buffering capacity, CO₂ excretion *in vivo* still occurs primarily via the red cell, despite the rate-limiting constraint of anion exchange, because of the high intracellular proton availability. While whole blood buffering capacity in rainbow trout is about -9.7 mmol L⁻¹ pH unit⁻¹ (at 25% haematocrit) (Wood et al., 1982), this value takes into account the low plasma β (-2.6 mmol L⁻¹ pH unit⁻¹) and red cell buffering capacity is therefore presumably much higher (Tufts and Perry, 1998). Moreover, CO₂ excretion in rainbow trout is tightly linked to O₂ uptake through the release of Bohr protons from haemoglobin during oxygenation (Perry and Gilmour, 1993; Brauner et al., 1996, see reviews by Brauner and Randall, 1996, 1998). It appears that these proton sources are of overwhelming importance to CO₂ excretion *in vivo*, in that although plasma HCO₃⁻ dehydration rates *in vitro* were elevated by as much as 20-75% by increasing buffering capacity, the addition of bovine CA to provide an extracellular site for HCO₃⁻ dehydration *in vivo* in trout with elevated plasma buffering capacity had only a small impact on CO₂ excretion.

Acknowledgements

This study was supported by NSERC of Canada research and equipment grants to KMG. Dr. Steve Perry is thanked for his helpful comments on the manuscript.

References

- Bidani, A. 1991. Analysis of abnormalities of capillary CO₂ exchange *in vivo*. J. Appl. Physiol. 70:1686-1699.
- Bidani, A. & Heming, T.A. 1991. Effects of perfusate buffer capacity on capillary CO₂-HCO₃⁻-H⁺ reactions: theory. J. Appl. Physiol. 71:1460-1468.

- Boutilier, R.G., Heming, T.A. & Iwama, G.K. 1984. Appendix: Physicochemical parameters for use in fish respiratory physiology. In: W.S. Hoar & D.J. Randall (Eds.). *Fish Physiology*. Academic Press, London, pp. 403-430.
- Brauner, C.J. 1995. An analysis of the transport and interaction of oxygen and carbon dioxide in fish. Ph.D. thesis, University of British Columbia. 148pp.
- Brauner, C.J., Gilmour, K.M. & Perry, S.F. 1996. Effect of haemoglobin oxygenation on Bohr proton release and CO₂ excretion in the rainbow trout. *Respir. Physiol.* 106:65-70.
- Brauner, C.J. & Randall, D.J. 1996. The interaction between oxygen and carbon dioxide movements in fishes. *Comp. Biochem. Physiol.* 113A:83-90.
- Brauner, C.J. & Randall, D.J. 1998. The linkage between oxygen and carbon dioxide transport. In: S.F. Perry & B.L. Tufts (Eds.). *Fish Respiration*. Academic Press, San Diego, pp. 283-319.
- Cameron, J.N. & Polhemus, J.A. 1974. Theory of CO₂ exchange in trout gills. *J. exp. Biol.* 60:183-194.
- Gilmour, K.M., Perry, S.F., Bernier, N.J., Henry, R.P. & Wood, C.M. Extracellular carbonic anhydrase in dogfish, *Squalus acanthias*: A role in CO₂ excretion. *Respir. Physiol.*, submitted.
- Gilmour, K.M., Randall, D.J. & Perry, S.F. 1994. Acid-base disequilibrium in the arterial blood of rainbow trout. *Respir. Physiol.* 96:259-272.
- Heming, T.A. & Bidani, A. 1992. Influence of proton availability on intracapillary CO₂-HCO₃⁻-H⁺ reactions in isolated rat lungs. *J. Appl. Physiol.* 72:2140-2148.
- Julio, A.E., Desforges, P.R. & Perry, S.F. 2000. Apparent diffusion limitations for CO₂ excretion in rainbow trout are relieved by injections of carbonic anhydrase. *Respir. Physiol.* 121:53-64.

- Lessard, J., Val, A.L., Aota, S. & Randall, D.J. 1995. Why is there no carbonic anhydrase activity available to fish plasma? *J. exp. Biol.* 198:31-38.
- Malte, H. & Weber, R.E. 1985. A mathematical model for gas exchange in the fish gill based on non-linear blood gas equilibrium curves. *Respir. Physiol.* 62:359-374.
- Perry, S.F. 1986. Carbon dioxide excretion in fishes. *Can. J. Zool.* 64:565-572.
- Perry, S.F., Brauner, C.J., Tufts, B.L. & Gilmour, K.M. 1997. Acid-base disequilibrium in the venous blood of rainbow trout (*Oncorhynchus mykiss*). *Experimental Biology Online* 2, 1.
- Perry, S.F. & Gilmour, K.M. 1993. An evaluation of factors limiting carbon dioxide excretion by trout red blood cells *in vitro*. *J. exp. Biol.* 180:39-54.
- Soivio, A., Nynolm, K. & Westman, K. 1975. A technique for repeated sampling of the blood of individual resting fish. *J. exp. Biol.* 62:207-217.
- Swenson, E.R., 1990. Kinetics of oxygen and carbon dioxide exchange. In: R.G. Boutilier (Ed.). *Advances in Comparative and Environmental Physiology*. Springer-Verlag, Berlin, pp. 163-210.
- Thomas, S. 1994. Extracorporeal circulation. In: P.W. Hochachka & T.P. Mommsen (Eds.). *Biochemistry and molecular biology of fishes: Analytical Techniques*. Elsevier, Amsterdam, pp. 161-167.
- Tufts, B.L. & Perry, S.F. 1998. Carbon dioxide transport and excretion. In: S.F. Perry & B.L. Tufts (Eds.). *Fish Respiration*. Academic Press, San Diego, pp. 229-281.
- Wood, C.M., McDonald, D.G. & McMahon, B.R. 1982. The influence of experimental anaemia on blood acid-base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* 96:221-237.

Wood, C.M. & Munger, R.S. 1994. Carbonic anhydrase injection provides evidence for the role of blood acid-base status in stimulating ventilation after exhaustive exercise in rainbow trout. *J. exp. Biol.* 194:225-253.

Wood, C.M. & Perry, S.F. 1991. A new *in vitro* assay for carbon dioxide excretion by trout red blood cells: effects of catecholamines. *J. exp. Biol.* 157:349-366.

FINE STRUCTURE AND Na^+ , K^+ -ATPASE ACTIVITY
IN GLASS EEL GILLS
DURING TRANSITION TO FRESHWATER

Assunção Santos
Center of Marine and Environmental Research (CIMAR)
and Institute of Biomedical Sciences Abel Salazar, University of Porto
Lg. Prof. Abel Salazar 2, 4099-003 Porto, Portugal,
Phone: +351-22-206 22 69, Fax: +351-22-206 22 32,
E-mail: assantos@icbas.up.pt

Mário Sousa¹, Elsa Oliveira¹ and João Coimbra²
¹Lab. of Cell Biology, and ²Lab. of Applied Physiology and
CIMAR, Institute of Biomedical Sciences Abel Salazar, University of Porto

EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

Ionocytes of fish gills are responsible for acid-base and osmo-regulation (Perry, 1997). They adapt to external conditions, undergoing proliferation and hypertrophy in media with increasing salinities. There are two main types of ionocytes, A-cells (in fishes adapted to sea water) and B-cells (in fishes inhabiting freshwater). In the present study we describe the fine structure and cytochemistry of ionocytes in gills of the glass eel *Anguilla anguilla*, which seasonally leave sea water and migrate into freshwater.

Materials and Methods

Specimens were collected during their seasonal migration from sea water to freshwater (river Minho, North frontier of Portugal), classified and processed for transmission electron microscopy and ultrastructural detection of Na^+ , K^+ -ATPase activity (Mayahara et al., 1980).

Results

Both type-A and B cells were observed. B-cells (Figure 1) were more numerous, larger, and separated from the external medium (L) by a layer of pavement cells (p). A-cells (Figure 2) were smaller, and in direct contact with the external milieu, showing a very dense cortical region due to the presence of a microfilament terminal web. They exhibited microvilli (arrows) that possess a dense cell coat. Both cells exhibited numerous mitochondria (m) and an extensive smooth endoplasmic reticulum (SER) tubular system. A-cells showed more SER vesicles in the apex than in the basal half, with the contrary occurring in B-cells. Na^+, K^+ -ATPase activity was present in both cell types, but the reaction was weaker in B-cells (Figure 1) than in A-cells (Figure 2).

The reaction product was consistently found over all the SER, being particularly intense in the apical region. Staining was totally inhibited in the absence of substrate and K^+ ions, or in the presence of 10 mM NaF and vanadate, but was not abolished with 2.5 mM levamisole. 10 mM ouabain inhibited the apical reaction, whereas 20 mM inhibited completely the enzymic activity.

Conclusions

We here show that A- and B-ionocytes can be easily distinguished by their fine structure. During early adaptation to freshwater, A-cells decreased in number, were smaller, the apical surface was kept in direct contact with the external medium, and exhibited higher and specific, ouabain-sensitive Na^+, K^+ -ATPase activity. On the contrary, B-cells appeared larger, in higher numbers, were separated from the external medium by pavement cells, and showed a less intense enzymic reaction.

References

- Mayahara, H., Fujimoto, K., Ando, T. and K. Ogawa. 1980. A new one-step method for the cytochemical localization of ouabain-sensitive, potassium-dependent p-nitrophenylphosphatase activity. *Histochem.* 67:125-138.

Perry. S.F. 1997. The chloride cell: structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol.* 59:325-347.

Acknowledgements

Mr. João Carvalheiro for the iconographic work, and grants from FCT.

**ION TRANSPORT IN RIO NEGRO FISH:
DEALING WITH THE DOUBLE WHAMMY**

Richard J. Gonzalez
Dept. of Biology, University of San Diego
5998 Alcalá Park
San Diego, CA 92110
ph: (619) 260-4077
fax: (619) 260-6804
email: gonzalez@acusd.edu

EXTENDED ABSTRACT ONLY – DO NOT CITE

The Rio Negro, a major tributary of the Amazon River, drains extremely mineral poor soils and is very dilute. Typical cation concentrations of the river in $\mu\text{mol L}^{-1}$ are: $\text{Na}^+ = 16.5 \pm 5.3$, $\text{K}^+ = 8.2 \pm 2.7$, $\text{Ca}^{2+} = 5.3 \pm 1.6$, $\text{Mg}^{2+} = 4.7 \pm 1.4$ (Furch 1984); small forest streams that feed the Rio Negro can be even more dilute. These waters have a very low buffering capacity and the presence of organic acids from decaying vegetation (giving the water its tea color, and name), make it acidic ($< \text{pH } 4.5$). Despite these seemingly harsh conditions, estimates indicate that almost twice as many species inhabit the Rio Negro as all the waters of North America.

Ion poor water of low pH such as that found in the Rio Negro pose a dual challenge for ion transport in fish. Both the scarcity of salts and low pH inhibit ion uptake (a double whammy for ion transport). Since the rate of ion uptake is concentration dependent, extremely low levels of Na^+ and Cl^- in the water can reduced uptake simply because of the extreme scarcity of these salts. Alternatively, low pH can inhibit Na^+ uptake since transport across the apical membrane of the gill epithelium requires the extrusion of H^+ . The inhibition of Na^+ transport can then lead to the inhibition of Cl^- uptake. (Perry and Randall 1982). The theoretical low pH limit for active ion uptake is believed to be about pH 4.0 (Gonzalez 1996). In San Diego and in Brasil colleagues and I, have been examining the ion transport capabilities of Rio Negro fishes to determine what specializations they possess that allow them to successfully ionoregulate and inhabit this river despite the harsh conditions.

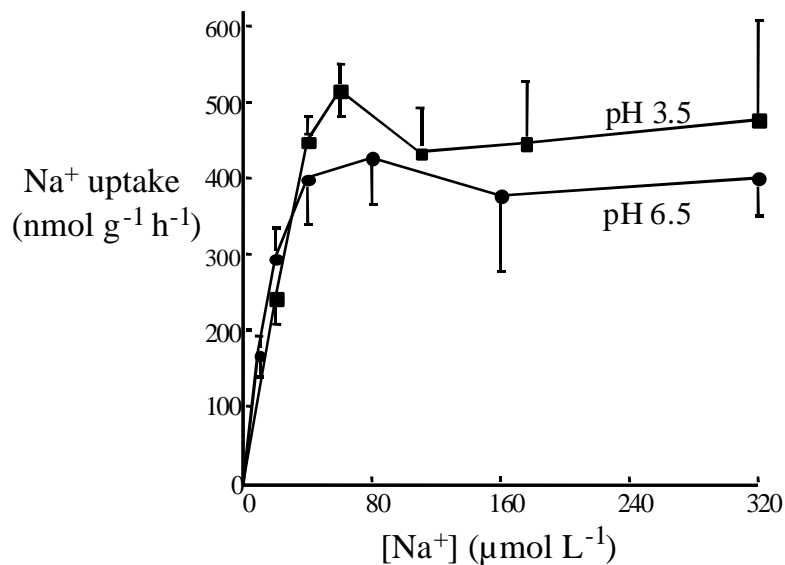
Our measurements of Na⁺ transport in relation to water Na⁺ concentration have shown that many Rio Negro species possess high affinity, high capacity transporters (Table 1). Among

Table 1. Kinetic parameters of Na⁺ transport in some Rio Negro fishes.

Species	K_m (μmol L⁻¹)	J_{max} (nmol g⁻¹ h⁻¹)
Neon tetras	12.9 ± 5.8	448.2 ± 43.5
Cardinal tetras	53.7 ± 7.8	773.0 ± 38.2
Blacksirt tetras	27.7 ± 2.7	691.3 ± 19.9

those tested, neon tetras have the lowest K_m value recorded for freshwater fish (Gonzalez and Preest 1999). These characteristics are clearly adaptive for operation in ion-poor waters. They allow the species to take up salts at high rates even when water concentrations are very low (Gonzalez et al. 1997). Interestingly there appears to be a phylogenetic component to these characteristics. The three species listed above are characins, but several species of cichlids have much lower affinities.

Figure 1. The effect of water pH on the relationship between rate of sodium uptake and water Na⁺ concentration in neon tetras.



Our examination of pH effects on ion transport revealed that, in general, Rio Negro species possess a reduced sensitivity to low pH. Most interesting, however, is the finding that one species, neon tetras, possess an ion transport mechanism that is completely insensitive to pH (Fig. 1). Kinetic analysis of Na⁺ transport at pH 6.5 and 3.25 yielded identical K_m and J_{max} values (Cl⁻ transport was also found to be pH insensitive). In fact, Na⁺ uptake of neon tetras was still uninhibited at pH 3.25, an almost 2000-fold increase in H⁺ concentration, relative to pH 6.5, and the lowest pH at which uptake has ever been observed.

To probe the nature of the neon tetras' pH insensitive transport mechanism we measured the rate of Na⁺ uptake in the presence of various pharmacological agents that block different types of transporters. Amiloride analogues, such as DMA, HMA, MIA, and EIPA, that block Na⁺/H⁺ antiporters had effects ranging from 0 to 40% inhibition. Na⁺ channel blockers like Benzamil and Phenamil

had no effect on Na⁺ transport, H⁺-ATPase inhibitor vanadate lowered uptake by 25%. These results do not offer strong support for the presence of either Na⁺/H⁺ antiporters or H⁺-ATPase/Na⁺ channel arrangements in neon tetras.

References

- Furch K. 1984. Water chemistry of the Amazon basin: the distribution of chemical elements among freshwaters. Pp. 167 - 199 in H. Sioli, ed. The Amazon. Limnology and Landscape Ecology of a Mighty Tropical River and its Basin. Dr. W. Junk Publishers, Dordrecht, Netherlands.
- Lin, H. & D. Randall. 1991. Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. J. Exp. Biol. 161: 119 - 134.
- Gonzalez R.J. 1996. Ion regulation in ion poor waters of low pH. Pp. 7 - 22 in A.L. Val, V.M.F. de Almeida-Val, and D.J. Randall, eds. Physiology and Biochemistry of the Fishes of the Amazon. National Institute for Amazon Research, Manaus.
- Gonzalez R.J., V.M. Dalton, and M.L. Patrick. 1997. Ion regulation in ion-poor, acidic water by the blackskirt tetra (*Gymnocorymbus ternetzi*), a fish native to the Amazon River. Physiological Zoology 70: 428 - 435.
- Gonzalez R.J., and M.R. Preest. 1999. Ionoregulatory specialization for exceptiona tolerance of ion-poor, acidic waters in the neon tetra (*Paracheirodon innesi*). Physiological Zoology 72: 156 - 163.
- Perry,S.F. and D.J. Randall. 1981. Effects of amiloride and SITS on branchial ion fluxes in rainbow trout, *Salmo gairdneri*. J. Exp. Zool. 215: 225 - 228.