

**ERYTHROCYTIC ORGANIC PHOSPHATES**  
**IN THE RED BLOOD CELLS**  
**OF FISH OF THE AMAZON**

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**Abstract**

Fish of the Amazon are exposed to continuous changes in dissolved oxygen. They respond to these changes by a wide spectrum of compensatory and respiratory mechanisms directed towards facilitating both oxygen uptake and oxygen unloading to tissues. Adjustment of erythrocytic levels of organic phosphates is one of these mechanisms and is improved as soon as the animal detects any change in dissolved oxygen. These phosphates are negative modulators of haemoglobin oxygen affinity and changes in their concentration result in changes in oxygen transfer to tissues. The present paper examines the diversity of organic phosphates in the erythrocytes of fish of the Amazon, their effects on haemoglobin oxygen affinities and the time course of their adjustments when the animals are exposed to deep hypoxia, estimating the effects of these changes on  $P_{50}$  over time of exposure.

**Introduction**

According to Randall *et al.* (1981) hypoxia and anoxia have been common constraints during the evolutionary history of tropical fresh waters. In parallel to seasonal changes in dissolved oxygen, the fish of the Amazon are regularly exposed to variations in the concentration of this gas that tend to occur in a very short period of time. In many lakes of the Amazon, oxygen levels may drop to zero at night and reach over-saturated levels at noon the very next day. Short-term changes in dissolved oxygen force, indeed, rapid respiratory adjustments

directed towards enhancing oxygen transfer to tissues. These adjustments include behavioural, morphological, physiological and biochemical characteristics. Part of these adjustments may require also secondary adjustments, as is the case of oxygen unloading to tissues when the animals migrate out of unfavourable environments (Val and Almeida-Val, 1995).

The physiological importance of red cell phosphates for oxygen transfer from the environment to tissues has been recognized since the classical papers by Benesch and Benesch (1967) and Chanutin and Curnish (1967). Fish haemoglobins, with few exceptions, are sensitive to organic phosphates. They bind to haemoglobin decreasing oxygen affinity, i.e., the higher the proportion of haemoglobin to organic phosphate the higher the  $P_{50}$  (the lower the affinity to  $O_2$ ). Because fish experience both changes in environmental oxygen availability and in their oxygen needs, adjustment of the proportion of Hb:organic phosphates is of significant adaptive advantage.

The availability of organic phosphates for reacting with haemoglobin can be altered by complex formation with other components. In the red blood cells they can readily complex with magnesium, for example, what greatly reduces their effect on Hb- $O_2$  affinity. Compared to marine fish, the red blood cells of fish of the Amazon have low levels of divalent cations, in particular magnesium (Mangum *et al.*, 1978), and this, although related to the Amazon ion-poor waters, may be important for oxygen transfer.

The present paper reviews the occurrence of the major organic phosphates in the erythrocytes of fish from a phylogenetic perspective and analyses the effect of the adjustment of their erythrocytic concentration on  $P_{50}$  over time in fish of the Amazon exposed to deep hypoxia.

#### *Diversity and phylogenetic occurrence*

ATP and GTP are the most common organic phosphate found in fish erythrocytes. In addition to these compounds, several other organic phosphates have been described, such as 2,3DPG in *Hoplosternum littorale*, IPP in *Arapaima gigas*,  $IP_2$  and UTP in *Lepidosiren paradoxa* (see Val, 2000 for references). The effect of organic phosphate on haemoglobin oxygen affinity of fish decreases in the following order: IHP(IPP)>GTP>ATP>2,3DPG.

The most primitive fish of the Amazon are the freshwater stingrays (Potamotrigonidae). The red blood cells of these animals have both ATP and GTP, both of them modulators of Hb-O<sub>2</sub> affinity. The following group, the Osteoglossomorpha, includes the air-breather *Arapaima gigas* and two species of *Osteoglossum*, *O. bicirrhosum* and *O. ferreirai*. All these three species have ATP and GTP in their erythrocytes. In addition to these compounds, the red blood cells of adult *Arapaima* present high levels of inositol pentaphosphate (IPP), a compound also described in the erythrocytes of two marine elasmobranches (Borgese and Nagel, 1978). The presence of IP<sub>2</sub> and UTP in the red blood cells of Dipnoi (Bartlett, 1978) possibly represents a novelty; no other fish species so far analysed have these compounds in their red blood cells. Among the Osteichthyes, the appearance of 2,3DPG in the red blood cells of *Hoplosternum* and *Cyathlasoma* is of evolutionary significance. 2,3DPG was first described in fish erythrocytes by Rapoport and Guest (1941) in *Amiurus* and *Micropterus*.

These data show that ATP, GTP and 2,3DPG, the three main modulators of Hb-O<sub>2</sub> affinity, appeared among fish and have been conserved during the evolution of vertebrates and are all found in the red blood cells of Tetrapoda. Inositol pentaphosphate, also appeared among fish, and is currently present in red cells of birds and turtle.

#### *Erythrocytic organic phosphates in fish exposed to deep hypoxia*

A decrease in the levels of ATP and GTP in the red blood cells of fish experiencing hypoxia has been reported for all Amazonian species so far analysed. Because these animals face rapid changes in dissolved oxygen in nature, the immediate question is how fast they can adjust the levels of these modulators, and so Hb-O<sub>2</sub> affinity, improving oxygen transfer to tissues. Previous analysis of representatives of the species *Osteoglossum bicirrhosum*, *Liposarcus pardalis*, *Mylossoma duriventris* and *Pygocentrus nattereri* indicate that they are able to reduce the levels of GTP down to 50% of the initial values within the first 15 minutes under hypoxia and to reach a new steady state (ca 75% reduction in GTP levels) after 30 minutes at acute hypoxia. Similar results have been observed for juveniles of *Colossoma macropomum*. Reduction of ATP levels, however, was a much slower process. Reduced levels of ATP are observed only after 60 minutes at deep hypoxia. Recovery of NTP levels is also a fast process.

Anaemia caused either by reduction in circulating red blood cells or by conversion of haemoglobin to methaemoglobin also results in internal hypoxia. In contrast to environmental hypoxia, anaemia elicits an increase in ATP and GTP in the red blood cells (Val *et al.*, 1994). The time course for the adjustments of ATP and GTP in anaemic animals is also a rapid process.

These two situations suggest that fish can actively adjust the levels of ATP and GTP in the red blood cells to increase either oxygen uptake at the gills or oxygen unloading to tissues. In both cases, the mechanisms controlling the levels of ATP and GTP in the red blood cells are unknown.

#### *Erythrocytic phosphates in fish exposed to hyperoxia*

Fish of the Amazon are regularly exposed to high levels of dissolved oxygen. Surprisingly, the effect of hyperoxia on erythrocytic levels of organic phosphates in fish is known for a reduced number of species and the data is conflicting. Long-term exposure of *Colossoma macropomum* resulted in a decrease in NTP levels, similar to the result reported by Wood *et al.* (1975) for *Pleuronectes platessa*. These results contrast with that observed for *Astronotus ocellatus* that exhibited no changes in NTP levels after 15 days of exposure to  $P_{O_2}=300$  mmHg (Marcon and Val, 1996). Short-term exposure to hyperoxia (6 hours), however, elicited an inverse response. Specimens of *Serrasalmus rhombeus* and *Oxydoras niger* presented a significant increase in GTP levels and no changes in ATP. In general, fish exposed to hyperoxia exhibit a decrease in ventilation volume as the  $P_{O_2}$  increases. This response would help to keep tissue oxygen supply under control; thought high levels of oxygen at the tissue level would result in severe disturbances. More data on the control of erythrocytic levels of GTP and ATP in animals exposed to hyperoxia are needed before a clear picture can be presented. It would be reasonable, however, to postulate that an increase in the levels of Hb-O<sub>2</sub> modulators would help to reduce oxygen unloading in the tissue in animals exposed to short-term hyperoxia.

#### *Effect of ATP and GTP on Hb-O<sub>2</sub> affinity*

Changes in erythrocytic cofactor levels in tambaqui affect Hb-O<sub>2</sub> affinities what safeguards oxygen loading at the gills. GTP, as in several other fish species of the Amazon, is stronger than ATP in decreasing the Hb-O<sub>2</sub> affinity in tambaqui. At pH 7.0, P<sub>50</sub> values change from 0.71 for stripped haemolysate to 6.17 mm Hg when 1mM GTP is added to the sample (1:1 Hb:GTP). An intermediary P<sub>50</sub>

figure was observed in the presence of 1mM ATP. Interestingly, this effect of GTP on Hb-O<sub>2</sub> affinity (compared to ATP) is also associated with faster changes of its intraerythrocytic level when the animal is exposed to deep hypoxia, which results in an almost immediate adjustment of oxygen transfer to tissues according to environmental oxygen availability. This rapid adjustment of ATP and GTP levels is important when the animal is skimming the water surface and is exposed to higher temperatures.

### **Concluding Remarks**

The analysis of the erythrocytic phosphates in fish of the Amazon clearly indicates that all main modulators of Hb-O<sub>2</sub> affinity known to occur in the erythrocytes of advanced vertebrates have appeared early in the evolution, among fishes. All Amazonian fish species so far analysed exhibit a significant decrease in the levels of GTP and ATP within 30 minutes of exposure to hypoxia. Short-term exposure to hyperoxia, however, elicits an increase in ATP and GTP. This adjustment helps to reduce oxygen unloading to the tissues in animals transiently exposed to hyperoxia.

### **Acknowledgements**

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**ORGANIC PHOSPHATE, pH AND ION REGULATION IN NORMOXIC  
AND HYPOXIC RED BLOOD CELLS OF AMAZONIAN FISH  
FOLLOWING ADRENERGIC STIMULATION.**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

Catecholamines are released into the blood of many fish species during periods of stress. In the red blood cells (RBC) of several teleosts, catecholamines bind to  $\beta$ -adrenoreceptors and activate  $\text{Na}^+/\text{H}^+$  exchange resulting in a suite of cellular events. These catecholamine induced changes ultimately lead to an increase in RBC volume,  $[\text{Na}^+]$  and pH and a reduction in NTP levels (Nikinmaa, 1990). The adaptive significance of these effects is to secure  $\text{HbO}_2$  transport during periods of oxygen stress by increasing  $\text{HbO}_2$  affinity and  $\text{O}_2$  transport capacity of the blood. While these effects are well characterized in many temperate fish species (Nikinmaa, 1990), relatively little is known about the diversity of this response among tropical fishes. A recent study indicated that the RBC adrenergic response is present in 2 of 4 species of characiformes but completely absent in 2 species of siluriformes (Val et al., 1998). In the present study, RBC

adrenergic responsiveness was investigated in 3 species of characiformes [ pacu (*Mylossoma duriventris*), red bellied piranha (*Pygocentrus nattereri*) and tambaqui (*Colossoma macropomum*)] and 2 species of osteoglossiformes [ arowana (*Osteoglossum bicirrhosum*) and pirarucu (*Arapaima gigas*)] to further investigate the ubiquity of this response.

### **Materials and Methods**

Pacu, piranha and arowana were captured by gill net in the Rio Solimoes and experiments were conducted on board the INPA Research Vessel *Amanai II* moored approximately 20 km upstream from Manaus, Brazil. Tambaqui and pirarucu were purchased from a local fish farm and experiments on these species were conducted at INPA. The red blood cells of the respective teleosts were rinsed several times in ice cold 10 mM HEPES buffered Cortland saline and left in the fridge overnight. The following morning the red cells were rinsed again and resuspended in this saline and split into four aliquots. Two aliquots were maintained in normoxia (air saturated) and 2 aliquots were maintained in hypoxia (N<sub>2</sub>) for 30 mins. A normoxic and a moderately hypoxic aliquot were each adrenergically stimulated (adrenaline = 10<sup>-5</sup> M) and maintained in the same gas composition for 60 mins. The other normoxic and hypoxic aliquots were left for 60 mins as controls. The adrenaline-induced effects on the pH gradient across the red cell (pH<sub>e</sub>-pH<sub>i</sub>), haematocrit (Hct) and mean cell haemoglobin concentration (MCHC) as well as organic triphosphate (NTP) levels and plasma Na<sup>+</sup> and K<sup>+</sup> concentrations were investigated.

### **Results and Discussion**

Two of the characins, pacu and tambaqui, exhibited adrenergically activated red cell Na<sup>+</sup>/H<sup>+</sup> exchange as indicated by changes in plasma [Na<sup>+</sup>], pH<sub>e</sub>-pH<sub>i</sub>, Hct and MCHC, that were more pronounced in hypoxia than normoxia. The response in tambaqui is consistent with that observed by Val et al., (1998). Adrenergic activation of red cell Na<sup>+</sup>/H<sup>+</sup> exchange was not apparent in the red bellied piranha (consistent with that observed in the black piranha, Val et al., 1998) or in the osteoglossids (arowana and pirarucu). Interestingly, all species tested exhibited a significant adrenaline-induced reduction in plasma K<sup>+</sup> concentration that was more pronounced in hypoxia than normoxia which has also been observed in the blood of trout (Nielsen and Lykkeboe, 1992). The only fish species that exhibited a reduction in red cell NTP levels associated with adrenergic stimulation was pacu, however, the degree of change was modest (<10%) compared with that observed in salmonids. The lack of adrenergically

mediated changes in red cell NTP levels under a similar experimental protocol have also been reported for other Amazonian fishes, including characins and catfishes (Val et al., 1998).

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**SWIMMING AND METABOLIC PERFORMANCE  
OF HIGH DESERT REDBAND TROUT**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

Redband trout are a native, loosely classified, subspecies of rainbow trout known variously as *Oncorhynchus mykiss newberii* and *Oncorhynchus mykiss gairdneri*. Though widely distributed throughout Oregon, Washington and Idaho, 11 redband trout populations have disappeared and 10 others are presently at risk (Nehlsen *et al.* 1993). It is widely believed that redband trout populations have declined as a result of poor land use practices that resulted in elevated stream temperatures. However, there is anecdotal evidence that redband trout commonly hold position in streams above 24 °C, and data on trout species from the Southwest indicate that the critical thermal maxima of trout from arid zones is in excess of 28 °C (Lee and Rinne, 1980). Before effective management plans for the redband trout can be developed, it is important to determine whether distinct population segments exist, and to characterize the effect of elevated stream temperatures on the physiology of this unique species. We measured swimming performance, metabolic parameters and thermal preference in two populations of redband trout from the High Desert Ecoregion of Oregon; both from streams with thermal regimes where daily fluctuations in water temperature can exceed 10° C. The first study area

was a “cold” stream site, the Little Blitzen River, which rarely experiences maximum summer temperatures above 18°C. The second study area, Bridge Creek, was a “warm” stream site where maximum summer temperatures typically reach or exceed 24°C.

## **Methods**

In order to minimize capture stress and injury, juvenile redband trout were collected by volunteer anglers using dry flies and barbless hooks, and kept in stream cages for 2-7 days prior to experimental use.

### *Experiment #1: 12 – 14 °C*

A modified critical swimming speed ( $U_{crit}$ ) test was performed stream-side using Blatzka type swim-tunnel respirometers (volume 6.8 l; Univ. of Waterloo) to determine the swimming and metabolic capacity of individual fish. In this protocol, the current velocity was increased by 10 cm s<sup>-1</sup> every 20 minutes until swimming speeds of 10 to 40 cm s<sup>-1</sup> were achieved, and by 5 cm s<sup>-1</sup> thereafter until the fish exhausted. At each swimming speed, oxygen consumption was measured for 6–10 minutes, beginning 3 minutes after swimming speed was increased.

### *Experiment #2: 24 °C*

The influence of temperature on routine metabolic rate was assessed at 0.5 bl s<sup>-1</sup> by measuring oxygen consumption as stream water temperature was increased (approx. 2° C per hour) from 12 °C to 24 °C. After routine levels of oxygen consumption were measured at 24 °C for each fish, fish were given a modified  $U_{crit}$  test, as described in Experiment #1.

For these experiments, statistical analyses comparing metabolic variables and swimming performance between and within groups were performed using ANCOVA, with body mass or length as the covariate.

### *Temperature Preference*

Thirty-six hours after transport to Portland State University (PSU), temperature preference was determined by placing fish in a thermal gradient belonging to Dr. L. Crawshaw. The thermal gradient was composed of 9 lanes (2.5 m long;

28 cm wide; 10 cm deep), each equipped with thermocouples every 25 cm. Temperature within each lane ranged from approx. 8 to 30 °C. Thermal preference was determined as the average temperature selected during the 4<sup>th</sup> experimental hour. Preliminary experiments revealed that selected temperature was not affected by longer acclimation periods. Preferred temperature was compared between the Bridge Creek and Little Blitzen fish using ANOVA.

### **Results and Discussion**

The metabolic power and swimming ability of these redband trout were comparable to those reported for other wild salmonids of similar size (Beamish, 1978, Brett and Glass, 1973). Trout from both populations swam well at 24 °C. However, test temperature had a differential effect on swimming performance and metabolism in these two populations:

- 1) trout from the Little Blitzen River, with a colder summer thermal history, had similar values for metabolic power and swimming performance at 12 – 14 and at 24 °C;
- 2) fish from Bridge Creek, with a warmer summer thermal history, had significantly lower values of metabolic power and swimming performance at 12 - 14 as compared with 24 °C; and
- 3) the swimming performance of Bridge Creek trout was significantly better than that of fish from the Little Blitzen River at 24 °C.

Although these data indicate that redband trout can tolerate short exposures to water temperatures up to 24°C, and suggest that thermal history can significantly influence the temperature sensitivity of swimming and metabolic performance, trout from both populations selected water temperatures of approx. 13°C.

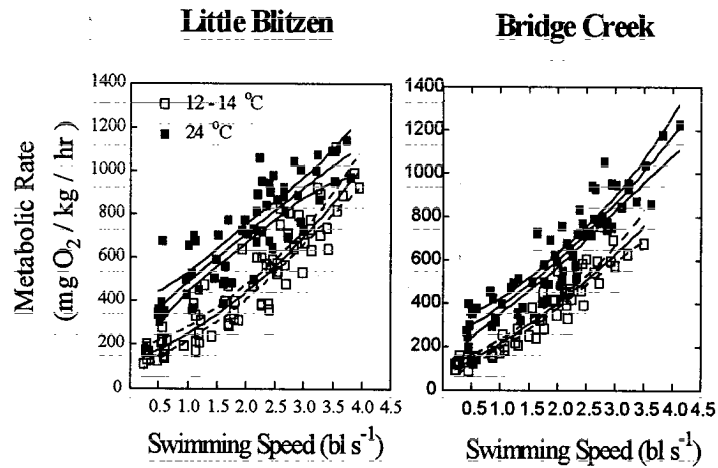


Figure 1. The relationship between swimming speed and metabolic rate for juvenile redband trout at 12 – 14 and 24 °C. Each data point represents an individual fish at a particular swimming speed. Experiments at 12 – 14 and 24 °C were performed on separate groups of fish. The lines drawn through each data set represent 2<sup>nd</sup> order regressions with 95% confidence limits.

**Table 1.** Resting  $\text{MO}_2$  and metabolic power were measured as  $\text{mg O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$  (\*) Indicates a significant difference ( $P < 0.05$ ) between streams, within each temperature; (#) indicates a significant difference ( $P < 0.10$ ) between streams, within each temperature; (+) indicates a significant difference ( $P < 0.05$ ) between temperatures, within each stream.

	Length (cm)	Resting $\text{MO}_2$	Metabolic Power	Ucrit ( $\text{cm s}^{-1}$ )
<b>BRIDGE</b>				
12-14 °C (N = 8)	20.3 ± 0.9*	122 ± 8 <sup>+</sup>	450.6 ± 44 <sup>+#</sup>	51 ± 3 <sup>+</sup>
24 °C (N = 7)	21.4 ± 0.9*	304 ± 28	633 ± 69	62 ± 3*
<b>BLITZEN</b>				
12-14 °C (N = 9)	17.6 ± 0.5	165 ± 12 <sup>+</sup>	661.5 ± 47	55 ± 3
24 °C (N = 9)	18.8 ± 0.4	383 ± 38	576.6 ± 47	49 ± 4

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**BIOCHEMICAL CHARACTERISTICS OF SERUM AND  
STRIATED MUSCLE FROM HIGH DESERT REDBAND TROUT**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

**Introduction**

Arid-land redband trout (*Oncorhynchus mykiss* ssp.) occur in southern Oregon, southwestern Idaho and northern Nevada. The United States Fish and Wildlife Service is considering the redband trout for threatened species status, in large part due to habitat degradation and elevated stream temperatures. However, data exist which both support and contradict the suggestion that elevated stream temperatures are responsible for declining redband trout populations. We have recently demonstrated that juvenile redbands can swim in excess of 3 body lengths per sec and have values for metabolic scope above 600 mg O<sub>2</sub>/kg/hr at 24°C. In addition, Rinne (1980) has shown that the critical thermal maximum of trout from southwestern arid regions exceeds 28° C. In contrast, both wild redband trout (Gamperl et al., unpublished data) and hatchery-reared rainbow trout (Peterson et al. 1979) have preferred temperatures below 15 °C.

The ability of redband trout to survive increased stream temperatures and harsh habitat conditions illustrates the remarkable plasticity of this salmonid and must be due to exceptional physiological and or biochemical adaptations. Before effective management plans for the redband trout can be developed, it is important to characterize the effects of elevated stream temperatures on this species and define the temperature optima for physiological function. Thus, the objective of this study was to examine whether habitat temperature influences

biochemical indices of energy metabolism in serum, axial white muscle, and the ventricle of redband trout.

## **Methods**

We focused on two stream populations of redband trout in southeastern Oregon during August of 1999. The Little Blitzen River, which rarely has maximum temperatures above 18°C, was designated as the “cold” stream. The second site, Bridge Creek, which is normally characterized by maximum temperatures above 24°C was selected as our “warm” stream. In both streams, the daily temperature fluctuation during summer can exceed 10°C. Fish were collected by hook and line, and allowed to recover in stream cages for at least 48 hrs. Fish were anesthetized, measured, weighed, and blood was collected from the caudal vein. Serum was isolated by centrifugation, the ventricle was excised, weighed, and a sample of white muscle was taken just ventral to the dorsal fin. All tissues were frozen rapidly in liquid nitrogen and transported to Idaho State University for biochemical analysis.

We measured serum osmolality using a vapor osmometer, and concentrations of triglycerides and free fatty acids by established spectrophometric techniques. Maximal activities of citrate synthase (CS, a mitochondrial marker of aerobic metabolism) and lactate dehydrogenase (LDH, a marker of anaerobic glycolysis) in white muscle were determined for whole tissue homogenates at 5°, 15°, and 25°C. CS activity in cardiac muscle was measured at 15°C. Sample means were compared by a one-way ANOVA and Tukey’s post hoc test. Statistical significance was established at  $P < 0.05$ . All values are expressed as mean  $\pm$  S.D.

## **Results**

Redband trout sampled from the Little Blitzen were significantly smaller than animals from Bridge Creek (Table 1). Relative ventricular mass was 28% greater in trout from the Little Blitzen, however, CS activity in cardiac muscle was not different between the two groups. Serum osmolality was significantly lower in trout from Bridge Creek. Concentrations of serum free fatty acids

	Length (cm)	Weight (g)	Relative Ventricle Mass (%)	Ventricle Citrate Synthase (U/g)	Serum Osmolality (mol/kg)	Serum FFA (mM)	Serum TG (mg/dl)
Blitzen (cold)	19.0 ± 2.3	74 ± 30	0.112 ± 0.019	19.6 ± 2.1	261 ± 14	1.1 ± 0.4	333 ± 63
Bridge (warm)	22.7 ± 4.2	144 ± 82	0.088 ± 0.012	18.6 ± 1.4	242 ± 23	0.8 ± 0.4	294 ± 73
P	0.007*	0.006 *	<0.001*	0.261	0.014*	0.094	0.137

Table 1. Physical and biochemical characteristics of redband trout from the Little Blitzen River and Bridge Creek.

n = 14 for all variables except ventricle citrate synthase (N = 8). FFA = free fatty acids, TG = triglycerides. \* Significant difference between groups.

(FFA) and triglycerides were similar between the two trout populations and comparable to plasma values reported previously for hatchery-reared rainbow trout (Rodnick and Williams 1999).

The activity of CS in white muscle was 1.6 to 2-fold higher in redband trout from the Little Blitzen at the temperatures examined. Conversely, LDH activity was 60% higher in white muscle of fishes from Bridge Creek. The corresponding thermal sensitivities ( $Q_{10}$  5°-25°C) of both CS (1.5-1.7) and LDH (1.7) were comparable between the two groups of fishes, suggesting the expression of similar enzyme isozymes at different environmental temperatures.

Table 2. Biochemical characteristics of axial white muscle from redband trout.

	Citrate Synthase			Lactate Dehydrogenase		
	5°C	15°C	25°C	5°C	15°C	25°C
Blitzen (cold)	2.1 ± 0.5	3.2 ± 0.7	4.6 ± 1.4	258 ± 85	474 ± 161	779 ± 283
Bridge (warm)	1.0 ± 0.2	1.6 ± 0.3	2.9 ± 0.9	433 ± 45	802 ± 96	1374 ± 286
P	<0.001*	<0.001*	0.014*	<0.001*	<0.001*	<0.001*

n = 8 for all assays. Maximal enzyme activities are expressed as units ( $\mu\text{mol}$  of substrate)/min/g tissue. \* Significant difference between groups.

### **Discussion and Conclusion**

Our results suggest that thermal history can selectively influence the characteristics of ventricle size, serum osmolality and the maximal activities of metabolic enzymes in white muscle of redband trout. A warm-adapted redband trout appears more dilute and may utilize higher rates of anaerobic metabolism during swimming activity than a cold-adapted redband trout. Conversely, the cold adapted trout appears to have a higher potential for aerobic metabolism and better maintenance of stroke volume at cold environmental temperatures. Together, these differences may help explain our recent findings that thermal history influences swimming and metabolic performance of redband trout.

### **Acknowledgements**

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**GLYCEROL PRODUCTION**  
**BY RAINBOW SMELT (*OSMERUS MORDAX*)**  
**AT SUB-ZERO TEMPERATURES IS DEPENDENT UPON**  
**BUT NOT CONTROLLED BY G3PDH ACTIVITY**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Rainbow smelt (*Osmerus mordax*) is a teleost that tolerates temperatures close to the freezing point of sea water through the use of proteins (Ewart and Fletcher, 1990) and glycerol (Raymond, 1992) as antifreeze agents. The lower the temperature, the higher the glycerol level such that at  $-2^{\circ}\text{C}$  serum glycerol may approach 400 mM (Raymond, 1992). Although effective at lowering the freezing temperature glycerol is lost at a rate that may exceed 10% of glycerol stores per day (Raymond, 1993). Therefore, there must be vigorous glycerol synthesis at very low temperatures.

Liver is an active site of glycerol synthesis and one source of carbon for glycerol is probably dietary amino acids that are degraded to glyceraldehyde 3-phosphate (G3P) (Raymond and Driedzic, 1997; Driedzic et al., 1998). It appears that carbon flow is  $\text{G3P} \rightarrow \text{dihydroxyacetone-P (DHAP)} \rightarrow \text{glycerol-3-P} \rightarrow \text{glycerol}$ . Blood glycerol levels and liver enzyme activity levels were determined in rainbow smelt, Atlantic tomcod (*Microgadus tomcod*) and smooth flounder (*Liposetta putmani*). All animals were sampled simultaneously from sub-zero water temperatures. Blood glycerol was 109, 0.29 and 0.17 mM in smelt,

tomcod, and flounder, respectively. Glycerol-3-phosphate dehydrogenase (G3PDH), the enzyme that catalyzes the conversion of DHAP to glycerol-3P was 156, 5.6 and 12.5  $\mu\text{mol min}^{-1} \text{g}^{-1}$  in smelt, tomcod, and flounder, respectively. Similarly, the activity of glycerol-3-phosphatase was approximately 3 to 6-fold higher in smelt than in tomcod or flounder liver (Driedzic et al., 1998). These findings may be considered in the framework of a large literature related to osmotic stress in yeast cells where hyperosmotic challenge results in glycerol production and increased expression of both G3PDH and glycerol-3-phosphatase (Norbeck and Blomberg, 1997).

More recently, smelt have been examined under controlled laboratory conditions to test the hypothesis that G3PDH is a key regulatory enzyme in glycerol production. Animals were collected in October and held in the laboratory under natural photoperiod conditions. All animals were fed frozen brine shrimp on a daily basis and populations ( $N = 5$ ) were sampled on Dec. 15, Jan. 11, Feb. 29, and Mar. 30. One group of animals was maintained at 4°C. A second group was held at ambient water temperature that decreased to 0.8°C by Jan. 11, to 0°C by Feb. 29, and to -0.8°C by Mar. 30.

Animals initially sampled in Dec. had plasma glycerol levels of  $80 \pm 26 \mu\text{mol ml}^{-1}$ . In smelt maintained at 4°C, plasma glycerol decreased to  $68 \pm 18$ ,  $31 \pm 20$ , and  $10 \pm 6 \mu\text{mol ml}^{-1}$ , on the sample dates stated above. In smelt living at ambient water temperature plasma glycerol levels were  $234 \pm 56$ ,  $217 \pm 33$ , and  $110 \pm 13 \mu\text{mol ml}^{-1}$ , on the sample dates stated above.

Animals initially sampled in Dec. had liver G3PDH activities of  $159 \pm 24 \mu\text{mol min}^{-1} \text{gm}^{-1}$  when assayed at 15°C. In smelt maintained at 4°C, liver G3PDH activities were  $84 \pm 14$ ,  $67 \pm 8$ ,  $78 \pm 10 \mu\text{mol min}^{-1} \text{gm}^{-1}$ , on the sample dates stated above. In smelt living at ambient water temperature liver G3PDH activities were  $103 \pm 30$ ,  $64 \pm 5$ ,  $67 \pm 6 \mu\text{mol min}^{-1} \text{gm}^{-1}$ , on the sample dates stated above. In both groups, liver G3PDH activities were lower following the initial sample date and there was no difference in enzyme activity between the two groups at any sample date.

In summary, smelt produce glycerol in response to a decrease in temperature that approaches 0°C. The activity of liver G3PDH is elevated relative to other species. High G3PDH activity in smelt liver is not induced by low temperature but instead is probably high, compared to other species, at all times. Although high activities of G3PDH are necessary for high rates of glycerol synthesis in

smelt, the induction of this enzyme is not a key element in the control process as it is in yeast cells that produce glycerol to increase osmotic pressure.

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**THE SNAKEHEAD *CHANNA ASIATICA***  
**PRODUCE AND STORES ALANINE**  
**DURING AERIAL EXPOSURE**

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**EXTENDED ABSTRACT ONLY-DO NOT CITE**

The snakehead, *Channa asiatica* is a valued food fish distributed over parts of Guangdong, China. It resides in slow-flowing streams and in crevices near riverbanks, and is a facultative air-breather, capable of terrestrial movement in an eel-like fashion. Upon aerial exposure in the laboratory, its ammonia excretion rate reduced to one-fourth that of the submerged control. Ammonia accumulated in the muscle, liver and plasma. It did not possess a complete ornithine-urea cycle and no carbamoyl phosphate synthetase activity was detected from its liver. Therefore, it was incapable of detoxifying ammonia to urea through the ornithine-urea cycle.

Instead, it accumulated alanine, asparagine and glutamine when exposed to the terrestrial condition. Different from the marble goby, *Oxyeleotris marmoratus*, (Jow et al., 1999) and the sleeper *Bostrichthyes sinensis* (Ip and Chew, unpublished results), the role of glutamine in *C. asiatica* appeared to be minor. In contrast, alanine content increased nine-fold in the muscle of *C. asiatica* during aerial exposure.

One of the primary sources of metabolic energy in carnivorous fishes is protein (Moon and Johnston, 1981). Amino acids released through proteolysis can be

either oxidized for energy production or converted to other utilizable forms by anabolic pathways. Certain amino acids (e.g. arginine, glutamine, histidine and proline) can be converted to glutamate. Glutamate can undergo deamination catalyzed by glutamate dehydrogenase, producing  $\text{NH}_4^+$  and  $\alpha$ -ketoglutarate (Campbell, 1991). The latter is then fed into the Krebs cycle. On the other hand, glutamate can undergo transamination with pyruvate, catalyzed by alanine aminotransferase (ALT), producing  $\alpha$ -ketoglutarate and alanine without the release of ammonia. If there is a continuous supply of pyruvate, transamination would facilitate the oxidation of carbon chains of some amino acids without polluting the internal environment with ammonia.

Under normal circumstances, the carbon chain of an amino acid can be completely oxidized to carbon dioxide and water through the Krebs cycle and the electron transport chain, producing ATP or its equivalent. For *C. asiatica* and the mudskipper *Periophthalmodon schlosseri* (Ip et al., submitted for publication) exposed to the terrestrial condition, the carbon chain may undergo only partial oxidation.  $\alpha$ -Ketoglutarate can be metabolized through portions of the Krebs cycle to malate. Malate can then be turned into pyruvate in the presence of malic enzyme. The presence of malic enzyme in the tissues of *C. asiatica* indicates that such a pathway is indeed possible. This strategy would cause a reduction in the efficiency of ATP production, as amino acids are not fully oxidized. However, for a fish having difficulties in excreting ammonia, this would be a useful strategy, because the pyruvate spun out of the Krebs cycle can combine with the amino group of glutamate to form alanine. This would allow the utilization of certain amino acids as energy sources without polluting the internal environment.

Aerial exposure did not affect the activities of ALT in the tissues of *C. asiatica*. An examination of the mass action ratio of the reaction catalyzed by ALT in the muscle of specimens exposed to the submerged or terrestrial condition revealed that isozymes of ALT might exist in this fish.

During the 48 h of aerial exposure, the reduction in nitrogenous excretion could be completely accounted for by the accumulation of various nitrogenous compounds, especially ammonia and alanine. This suggests that *C. asiatica* did not resort to suppressing protein and amino acid catabolism under such a condition. In this regard, it is different from the mudskipper *P. schlosseri*, which is capable of undergoing partial catabolism of certain amino acids and suppressing protein catabolism simultaneously (Lim et al., and Ip et al., submitted for publication).

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**THE LOACH *MISGURNUS ANGUILLICAUDATUS*  
REDUCES AMINO ACID CATABOLISM AND  
VOLATILIZES NH<sub>3</sub> DURING AERIAL EXPOSURE**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

The loach *Misgurnus anguillicaudatus* inhabits the rice field in Southern China. It encounters drought during summer and ammonia loading during agricultural fertilization. In the laboratory, aerial exposure led to decreases in its ammonia and urea excretion. However, it maintained an alkaline pH (>9) on its skin surface and can eliminate >25% of the ammonia excreted during aerial exposure as ammonia gas. This is the first fish known to be capable of doing so.

Ammonia accumulated to very high levels in the muscle and the liver. For most fishes, such high levels of ammonia in the tissue would disturb the nervous system, intracellular pH and integrity of membranes (Campbell, 1991). So, presumably, many physiological and biochemical processes in *M. anguillicaudatus* must be refractory to ammonia toxicity. Urea synthesis

through the ornithine-urea-cycle was not involved in ammonia detoxification in this fish. Very low activity of carbamoyl phosphate synthetase, which utilized glutamine as a substrate, was present in the liver mitochondria.

During the early phase of aerial exposure, *M. anguillicaudatus* accumulated alanine in its muscle. As suggested for the mudskippers (Ip et al., submitted for publication), *M. anguillicaudatus* might undergo partial amino acid catabolism, leading to the formation and storage of alanine. The process of alanine formation is not energy dependent; instead it provides a source of energy through partial catabolism of certain amino acids to support activities on land. This is not a detoxification mechanism, but a mechanism to avoid internal fouling by ammonia.

Upon prolonged exposure to the terrestrial condition, *M. anguillicaudatus* converted to glutamine formation as a means to detoxify the accumulating ammonia. So far, glutamine has only been found to play a role in ammonia detoxification in fish in response to high environmental ammonia concentration (Mommsen and Walsh, 1991), with the exception of the marble goby *Oxyeleotris marmoratus* (Jow et al, 1999). Formation of glutamine involves the hydrolysis of ATP, and it would make sense for it to take place when the fish is relatively inactive. However, different from the case of *O. marmoratus*, the glutamine accumulated in the body of *M. anguillicaudatus* could not account for the reduction in nitrogenous excretion in the terrestrial condition. Our results indicate that, similar to the mudskippers (Lim et al., submitted for publication), this loach was capable of reducing protein and/or amino acid catabolism during prolonged aerial exposure.

In its natural habitat, *M. anguillicaudatus* often encounters drought during summer. It moves actively on land until it encounters soft mud where it can bury itself through several strong wriggling action of the body. Hence, it is possible that it uses partial amino acid catabolism to fuel its short period of activities on land, and switches to the reduction in protein/amino acid catabolism, and formation of glutamine to detoxify the internally produce ammonia when it remains relatively inactive in the mud.

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**THE SLEEPER *BOSTRICHTHYES SINENSIS* (FAMILY ELEOTRIDAE)  
PRODUCES AND STORES GLUTAMINE  
DURING AERIAL EXPOSURE**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

*Bostrichthyes sinensis* is reputed as a good food fish rich in proteins, and is served as a tonic. It inhabits brackish water and finds its niche in the crevices of the river mouths of Shang Xi and Guangdong, China. In its natural habitat, it may encounter aerial exposure frequently during low tides, as it usually remains quiescent in the absence of water. Upon aerial exposure in the laboratory, the ammonia excretion rate decreased to one-fourth that of the submerged control. Different from the loach *Misgurnus anguillicaudatus* (Chew and Ip, unpublished results), *B. sinensis* was incapable of eliminating ammonia in gaseous form. The ammonia excretion rate of *B. sinensis* exposed to the terrestrial condition was ¼ of the submerged control, and ammonia was accumulated in the body of the fish. Although all the enzymes of the ornithine-urea cycle were present in the liver of this fish, the activity of hepatic carbamoyl phosphate synthetase using glutamine as a substrate was too low for the cycle to be functioning. Indeed, ammonia accumulated in the tissues was not converted to urea.

There are three other strategies which seem commonly adopted, singly or in combination, by fish to survive in the terrestrial condition:

reduction in proteolysis and/or amino acid catabolism to slow down the build up of ammonia.

partial catabolism of amino acid to alanine without releasing ammonia, and detoxification of internally produced ammonia to glutamine.

Strategies (1) and (2) are not detoxification mechanisms. Mudskippers (*Boleophthalmus boddarti* and *Periophthalmodon schlosseri*) adopt strategy (1) during aerial exposure in constant darkness (Lim et al, submitted for publication). However, *P. schlosseri* adopts strategies (1) and (2) when it is exposed to the terrestrial condition in a dark:light regime. Under such experimental conditions, it undergoes a reduction in amino acid catabolism in general with partial catabolism of certain amino acids to alanine to sustain activity on land (Ip et al, submitted for publication).

*B. sinensis*, did not produce and accumulate alanine when exposed to the terrestrial condition. Instead, it detoxified internally produced ammonia to glutamine (strategy 3 above) during the first 24 h of aerial exposure. During this period, the reduction in equivalent ammonia excreted amounts to 370  $\mu\text{mol}$ , which could be solely answered for by the accumulation of 441  $\mu\text{mol}$  (ammonia equivalent) as glutamine in the muscle. To our knowledge, glutamine has only been reported to play a role in ammonia detoxification in fish in response to high environmental ammonia concentrations (Mommsen and Walsh, 1991; Peng et al., 1998), with only one exception--the marble goby *Oxyeleotris marmoratus* (Jow et al, 1999).

Contrary to the production of alanine, as observed in the mudskipper *P. schlosseri* (Ip et al., submitted for publication) and the snakehead *Chana asiatica* (Chew and Ip, unpublished results), formation of glutamine is energetically expensive. *B. sinensis* and *O. marmoratus* are relatively inactive on land, and the reduced energy demand for muscular activity may provide them with the opportunity to exploit glutamine formation as a means to detoxify ammonia.

The accumulation of glutamine in *B. sinensis* leveled off after 48 h of aerial exposure. By the 72<sup>nd</sup> h of aerial exposure, there was a very great discrepancy between the reduction in excretion of ammonia equivalent (1109  $\mu\text{mol}$ ) and the retention of ammonia equivalent (458  $\mu\text{mol}$ ) as ammonia and urea. These results indicate that there might have been a reduction in proteolysis and amino acid catabolism when *B. sinensis* was exposed to the terrestrial condition for a long period (Strategy 1 above). Reduction in proteolysis and amino acid

catabolism is an effective strategy to slow down the internal build up of ammonia. If the rate of amino acid catabolism decreases and the rate of proteolysis remains unchanged, the steady state concentrations of free amino acids will increase. However, if the rate of proteolysis decreases to a greater extent than the rate of amino acid catabolism, the steady state levels of free amino acids will decrease. In this regard, *B. sinensis* is different from *O. marmoratus*, which is incapable of reducing proteolysis or amino acid catabolism in response to aerial exposure. *O. marmoratus* adopts strategy (3) for the whole period (72 h) of aerial exposure (Jow et al., 1999).

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**ALTERATIONS IN THE NITROGEN METABOLISM  
OF *Rivulus marmoratus* UNDER CONDITIONS OF PROLONGED  
AIR EXPOSURE -- ADAPTATIONS FOR SURVIVAL**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

**Abstract**

Mangrove killifish (*R. marmoratus*) are capable of surviving prolonged periods out of water (>1 month) and can tolerate relatively high levels of external ammonia (>10 mM NH<sub>4</sub>Cl). Under these extreme conditions alterations in nitrogen metabolism occur. During air exposure these fish continue to excrete both urea-N and ammonia, with a significant amount of the ammonia now being excreted through volatilization. Emersion also resulted in the accumulation of urea in the tissues after 4 days of exposure, while ammonia tissue levels did not change. Analysis of whole body tissue free amino acid levels revealed increases in both glutamine and alanine, whereas glutamate decreased significantly on days 1 and 4, but returned to control values by day 10. These findings demonstrate that *R. marmoratus* have evolved several strategies to prevent the toxic accumulation of ammonia, enabling them to survive under extreme environmental conditions.

## Introduction and Objectives

The killifish *Rivulus marmoratus* is a small cyprinodontid, hermaphroditic fish which inhabits tropical mangrove forests. *R. marmoratus* can tolerate an incredible range of environmental conditions including relatively high levels of external ammonia (>10 mM NH<sub>4</sub>Cl), and a range of salinities (0-68‰). They are also remarkable in their ability to survive out of water for extended periods of time (>1month). Thus, *R. marmoratus* may have evolved specialized mechanisms to deal with the problem of N-excretion under extreme conditions. Most teleosts are ammoniotelic (i.e. they excrete primarily ammonia), however ammonia is highly toxic and requires copious amounts of water for excretion. Thus, accumulation of ammonia under terrestrial conditions may occur and could be potentially poisonous. This study examined the mechanisms used by *R. marmoratus* to deal with the problem of nitrogen excretion on land.

## Materials and Methods

Fish were held in separate containers at 25°C in 17‰ artificial seawater, on a 12:12 hr photoperiod. Ammonia and urea excretion rates were measured in fish held in both water (control) and air (treatment), over a 10 day period. The amount of ammonia volatilized from fish held in air for 24 hours was quantified using the acid trap method described by Davenport and Sayer (1986). Ammonia and urea whole body tissue levels were measured after 1, 4, and 10 days of emersion according to the method of Wright *et al.* (1995). Whole body free amino acid levels (FAA) were also measured in air exposed fish over the same time period, using high performance liquid chromatography (HPLC) (Barton *et al.* 1995). The concentrations of ammonia and urea in water and tissue samples were determined using the methods of Ivancic and Degobbis (1984) and Rahmatullah and Boyde (1980), respectively.

## Results

Emersed fish continued to excrete both ammonia and urea, but at decreased rates (~50% and 36% respectively, of submerged rates), throughout the 10 days of exposure. Ammonia volatilization was found to occur, with almost half (~46%) of the total ammonia excreted through this method. The level of urea found in whole body tissue extracts was not significantly different after one day of emersion (compared to control values) but did increase significantly on day 4 and 10. Tissue ammonia levels did not change under emersed conditions. Analysis of tissue FAAs showed a significant increase in both glutamine and

alanine after one day of air exposure. A significant decrease in glutamate tissue levels was seen on days 1 and 4, with a return to control values occurring by day 10.

### Conclusions

*R. marmoratus* utilize several different strategies to deal with the problem of nitrogen excretion while in the terrestrial environment. They are able to continuously excrete N-wastes during emersion. Toxic ammonia is converted to urea, some of which is stored within the tissues of the fish during emersion, indicating a shift towards ureotelism. Even more fascinating is the incredible ability of this fish to volatilize a relatively large percentage of ammonia. Changes in tissue FAA levels suggest an alternative method of storing nitrogenous wastes during air exposure. *R. marmoratus* have adapted several different strategies which allow them to survive out of water. Studying these adaptations can provide valuable insight in the evolution of nitrogen metabolism and excretion from the aquatic to terrestrial environment.

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**THE DYNAMIC ROLE OF THE FISH GILL  
IN EXTREMELY ALKALINE ENVIRONMENTS**

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**Introduction**

Although salmonids are not usually subjected to extremely alkaline environments (pH > 9.0), they may have to withstand upward pH surges due to the photosynthetic processes of aquatic plants and algae (Murray and Zeibell 1984). There are also many permanently saline-alkaline lakes throughout Western North America, which has complicated efforts to stock these waters with salmonids such as the rainbow trout (*Oncorhynchus mykiss*) and other game fishes (Coleman and Johnson 1988; Wagner et al. 1997). A major complication of high pH exposure is that it leads to severe, sometimes lethal, reductions in plasma electrolytes (Yesaki and Iwama 1992). Thus, a major objective of this study was determine how ion uptake and loss across the rainbow trout gill was influenced by high pH exposure, and to determine how changes in the fine architecture of the gill allowed fishes to correct high pH induced ion disturbances. Highly alkaline environments also lead to pronounced blood acid-base disturbances (see Wilkie and Wood 1996 for review), which are frequently characterized by a combined respiratory alkalosis (reduced arterial P<sub>CO2</sub>) and metabolic acidosis (reduced arterial HCO<sub>3</sub><sup>-</sup> concentration). Thus, a second objective was to test the hypothesis that high pH-induced acid-base disturbances contributed to ion imbalances by interfering with gill mediated ion transport processes.

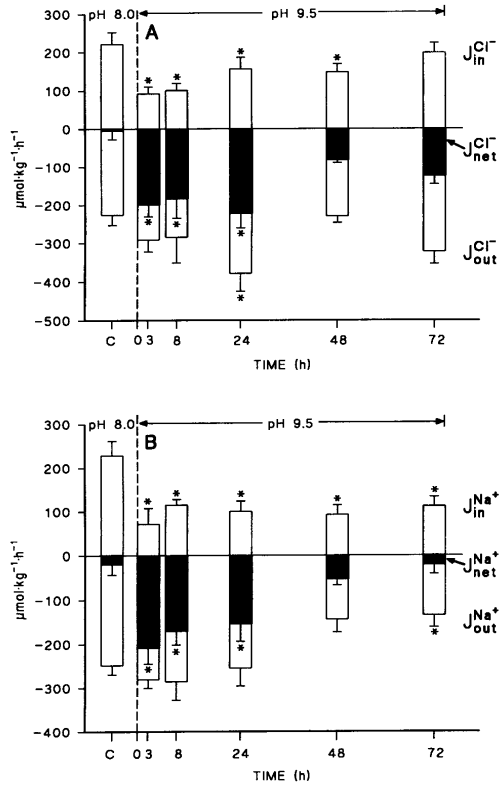
## Methods and Materials

To determine how disturbances to internal ion balance took place in alkaline environments, rainbow trout (N = 6-8) were exposed to pH 9.5 for 72 h, and radiotracers ( $^{22}\text{Na}^+$ ,  $^{36}\text{Cl}^-$ ) were used to monitor ion movements across the gills. The blood acid-base status of rainbow trout was also used to determine how high pH-induced alterations to internal metabolic acid ( $\text{H}^+$ ) and base ( $\text{HCO}_3^-$ ) concentrations influenced  $\text{Na}^+$  and  $\text{Cl}^-$  transport. To achieve this goal, we used the 2-substrate approach described by Goss and Wood (1991) to determine how ion uptake is influenced by a fish's internal acid-base status. A detailed ultrastructural analysis of the gills, using scanning and transmission electron microscopy, was also conducted to relate changes in the gill's fine architecture to altered patterns of ion movement. Since the chloride cells and pavement cells of gills appear to be the respective sites involved with  $\text{Cl}^-$  and  $\text{Na}^+$  uptake (Perry 1997), we hypothesized that high pH induced reductions in ion uptake, and eventual recovery of ion balance, would be closely correlated to changes in the ultrastructure of these cells. A complete description of the methods used in these experiments can be found in Wilkie et al. (1999) and Laurent et al. (2000).

## Results and Discussion

Altered ion balance at high pH was due to initial 60-70 percent reductions in  $\text{Na}^+$  influx ( $J_{\text{in}}^{\text{Na}}$ ) and  $\text{Cl}^-$  influx ( $J_{\text{in}}^{\text{Cl}}$ ), which lead to net losses of both ions (Figure 1). The decrease in  $\text{Cl}^-$  influx at high pH was partially due to transient 50 percent decreases in maximal  $\text{Cl}^-$  uptake rate ( $J_{\text{max}}^{\text{Cl}}$ ), which reflected a decrease in the number of  $\text{Cl}^-$  transport sites (Table 1). The development of a metabolic acidosis (Table 2), characterized by lower plasma  $\text{HCO}_3^-$  concentration, likely contributed to reduced  $J_{\text{in}}^{\text{Cl}}$  by limiting  $\text{HCO}_3^-$  supply to  $\text{Cl}^-/\text{HCO}_3^-$  exchangers on the apical membrane of branchial chloride cells. This interpretation was supported by the 2-substrate analysis, which indicated that there was a *real* decrease in the number of  $\text{Cl}^-$  transport sites on the gill after 10 h at high pH (Figure 2A). A decrease in the fractional surface area of the most active, highly villous chloride cells (CCs) on the gill epithelium after 8 h (Figure 3), also contributed to the decrease in  $J_{\text{max}}^{\text{Cl}}$  at that time.

A complete recovery of  $J_{\text{max}}^{\text{Cl}}$  after 72 h at high pH (Table 1) likely accounted for the recovery of  $\text{Cl}^-$  influx (Figure 1A). This recovery was paralleled by a two-fold increase in the total fractional surface area of branchial CC's (Figure 3), suggesting that the gill had actually increased its  $\text{Cl}^-$  transporting capacity.



**Figure 1.** Influx ( $J_{\text{in}}^{\text{ion}}$ ; upward facing bars), outflux ( $J_{\text{out}}^{\text{ion}}$ ; downward facing bars) and net movements ( $J_{\text{net}}^{\text{ion}}$ ; shaded bars) of (A)  $\text{Cl}^-$  and (B)  $\text{Na}^+$  across rainbow trout gills during exposure to pH 9.5 for 72 h. Asterisks demonstrate statistical significance ( $P < 0.05$ ) from control measurements at pH 8.0.

**TABLE 1.** Changes in the kinetic parameters of unidirectional Cl<sup>-</sup> and Na<sup>+</sup> uptake during exposure of rainbow trout to pH 9.5 for 72 h. Means  $\pm$  1 SEM (N = 6 - 7). C = control (pH 8.0) conditions.

pH	Time (h)	Chloride		Sodium	
		J <sub>max</sub> (μmol·kg <sup>-1</sup> ·h <sup>-1</sup> )	K <sub>m</sub> (μmol·L <sup>-1</sup> )	J <sub>max</sub> (μmol·kg <sup>-1</sup> ·h <sup>-1</sup> )	K <sub>m</sub> (μmol·L <sup>-1</sup> )
8.0	C	358.2 $\pm$ 39.9	311.0 $\pm$ 84.8	481.5 $\pm$ 53.1	88.5 $\pm$ 13.3
9.5	10	172.6 $\pm$ 30.2*	405.6 $\pm$ 116.3	155.5 $\pm$ 14.9*	66.2 $\pm$ 19.8
	24	285.8 $\pm$ 31.9	391.9 $\pm$ 51.7	259.2 $\pm$ 33.2*	188.3 $\pm$ 49.0
	72	297.3 $\pm$ 37.8	335.6 $\pm$ 58.1	322.6 $\pm$ 13.5*	375.2 $\pm$ 85.4*

Asterisks (\*) indicate significant differences from control (pH 8.0) values (p < 0.05).

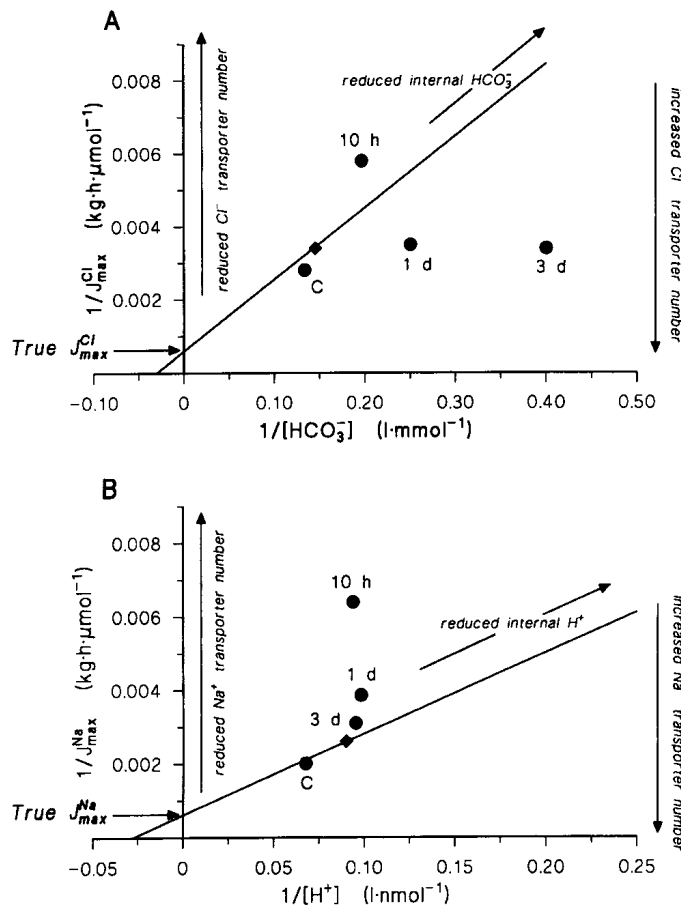
Data taken from Wilkie et al. (1999).

**TABLE 2.** Arterial Blood Acid-Base Status of Rainbow Trout at High pH (pH 9.5).

WATER	pH	Blood Acid-Base Status		
		[H <sup>+</sup> ] (nmolL <sup>-1</sup> )	P <sub>CO2</sub> (Torr)	[HCO <sub>3</sub> <sup>-</sup> ] (mmolL <sup>-1</sup> )
pH 8.0	7.83 $\pm$ 0.01	14.8 $\pm$ 0.3	3.07 $\pm$ 0.09	7.50 $\pm$ 0.30
pH 9.5				
8 h	7.97 $\pm$ 0.01*	10.7 $\pm$ 0.3*	1.43 $\pm$ 0.08*	5.04 $\pm$ 0.34*
24 h	7.99 $\pm$ 0.01*	10.2 $\pm$ 0.2*	1.12 $\pm$ 0.14*	4.08 $\pm$ 0.40*
72 h	7.98 $\pm$ 0.03*	10.5 $\pm$ 0.7*	0.68 $\pm$ 0.07*	2.58 $\pm$ 0.37*

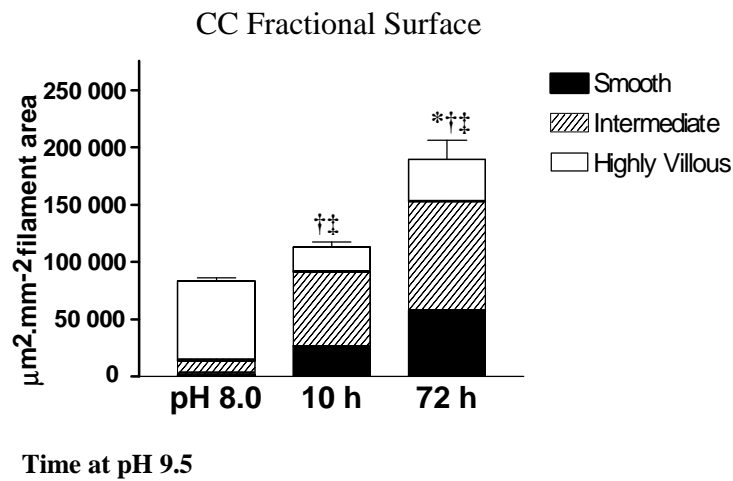
Asterisks (\*) indicate significant differences from pH 8.0 values (p < 0.05).

Data taken from Wilkie and Wood (1991).



**Figure 2.** Two-substrate kinetic analysis of ion uptake across rainbow trout gills at pH 8.0 or pH 9.5, based on methods used by Goss and Wood (1991). The analyses indicate the relative roles that internal substrate (counterion) availability and transporter number play in altering the respective apparent  $J_{max}^{Cl}$  and apparent  $J_{max}^{Na}$  for (A)  $\text{Cl}^-$  influx and (B)  $\text{Na}^+$  influx by rainbow trout. Respective arterial plasma  $\text{HCO}_3^-$  ( $[\text{HCO}_3^-]_a$ ) and  $\text{H}^+$  ( $[\text{H}^+]_a$ ) concentrations were taken from trout exposed to identical high pH conditions (Table 2; Wilkie and Wood 1991). The regression lines represent data collected by Goss and Wood

(1991), following imposition of different acid-base disturbances on the rainbow trout. The control data points of Goss and Wood (1991; diamonds) are indicated. Circles are the inverse of the apparent  $J_{\max}^{\text{Cl}}$  and  $J_{\max}^{\text{Na}}$  estimates (presented Table 1) plotted against corresponding inverse measurements of  $[\text{HCO}_3^-]_a$  and  $[\text{H}^+]_a$ . Upward or downward deviations (vertical arrows) away from the regression line represent *true* changes in transporter number, while changes in internal substrate availability are reflected by movements along or in parallel to the regression line (diagonal arrows).



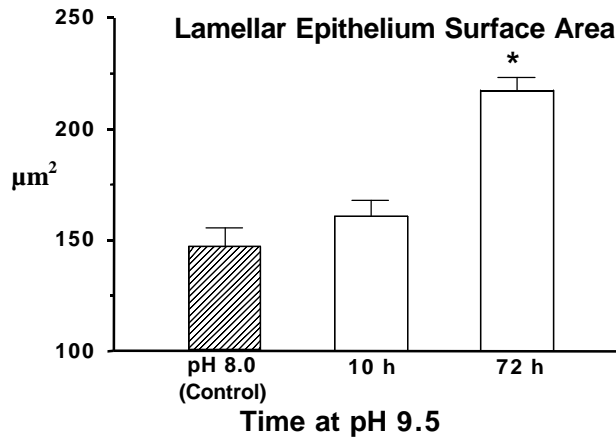
**Figure 3.** Alterations in the fractional chloride cell (CC) apical surface area on the filamental epithelium of rainbow trout gills at pH 8.0 or exposure to pH 9.5. Chloride cells were placed into one of three chloride cell classes according to the density of microvilli on their apical surface. Accordingly, the proportion of chloride cells with a smooth (no villi; solid region), intermediately villous (hatched region) or highly villous (open region) apical surface are illustrated. Values of fractional chloride cell apical surface area are based on the CC numerical density and individual CC apical surface area (not shown). Asterisks (\*) indicate statistically significant differences from pH 8.0 values for entire chloride cell populations, while daggers (†), double daggers (††), and stars (☆) indicate statistically significant differences from pH 8.0 values for smooth, intermediate and highly villous CC's, respectively

This observation was substantiated by the two-substrate analysis, which revealed an actual increase in  $\text{Cl}^-$  uptake capacity after 1 d and 3d (Figure 2A). Since  $\text{Cl}^-$  influx takes place via  $\text{Cl}^-/\text{HCO}_3^-$  exchange, greater branchial CC fractional surface area would also promote additional metabolic base excretion and therefore help to stabilize blood pH. Similar morphological observations in the Lahontan Cutthroat Trout of highly alkaline (pH 9.4) Pyramid Lake, Nevada (Wilkie et al. 1994), suggest that greater CC fractional surface area is an essential response that allows fishes to tolerate highly alkaline waters.

The 2-substrate analysis revealed that the respiratory alkalosis present in rainbow trout during high pH exposure (Table 2) limited  $\text{H}^+$  supply to the  $\text{Na}^+$  channel/proton transport system during the first 10 h of high pH exposure (Figure 2B). Thus, the respiratory alkalosis likely contributed to the reductions in  $J_{\text{in}}^{\text{Na}}$  commonly observed at high pH (Figure 1B). The restoration of net  $\text{Na}^+$  balance was partially explained by a progressive decrease in the  $\text{Na}^+$  permeability of the gill, as illustrated by significantly reduced  $\text{Na}^+$  outflux ( $J_{\text{out}}^{\text{Na}}$ ) after 72 h at high pH (Figure 1B). Although internal  $\text{H}^+$  supply continued to be limiting, the two-substrate analysis also revealed that there was a recovery of the *true*  $\text{Na}^+$  transport capacity of the gill by 3 d (Figure 2B). The interpretation that there was a *true* recovery of branchial  $\text{Na}^+$  transporting capacity was also supported by morphological examinations of the lamellar epithelium, which revealed that there was a greater density of microvilli on the pavement cells (Figure 4). As pavement cell membranes are where the putative  $\text{H}^+$ -pumps and possibly  $\text{Na}^+$  channels are located (Perry 1997), the 143 percent increase in the surface area of these cells likely contributed to the partial restoration of  $\text{Na}^+$  transport capacity that was observed after 3 d at high pH.

## Conclusions

Fish exposed to alkaline water experience acid-base disturbances that can directly impair ion uptake capability by limiting the supply of internal counterions such as  $\text{H}^+$  and/or  $\text{HCO}_3^-$ . Compensatory changes in gill architecture are essential, however, for allowing fishes such as the rainbow trout and the Lahontan cutthroat (*Oncorhynchus clarki henshawi*) trout to correct and maintain internal ion and acid-base balance in extremely alkaline environments.



**Figure 4.** Changes in the gill lamellar pavement cell (PVC) surface area in rainbow trout subjected to pH 8.0 (hatched bars) or pH 9.5 (open bars) for 10 h or 72 h. PVC surface area estimates take into account the topographical relief due to micro-villi and ridges, and were determined using an arbitrary reference surface area of 100  $\mu\text{m}^2$  (10  $\mu\text{m}$  X 10  $\mu\text{m}$ ) which would represent a completely smooth surface. Asterisks (\*) indicate statistically significant differences from pH 8.0 values.

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**ASSESSING CHEMICAL STRESS IN NEOTROPICAL FISH:  
A PHYSIOLOGICAL APPROACH**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

The impact of pollutants on aquatic biota can be studied by toxicity tests, which are used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms. Nevertheless, little research has been done on the impact of contaminants on tropical ecosystems. In order to extrapolate meaningful, relevant and ecologically significant results from aquatic toxicity tests, not only appropriate tests but also appropriate organisms should be used. Whenever possible, species should be studied which are indigenous to or representative of the ecosystem that may receive the impact. However, not many native fish species have been employed in toxicity tests in Brazil. Thus, there is a real lack of results concerning the sensitivity of Brazilian fish species to chemicals that are potential pollutants in tropical freshwater ecosystems, as well as the effects of these toxic agents on fish health. It was thus considered of interest to investigate the sublethal effects of pesticides (the herbicide Trifluralin and the insecticide Azodrin) and metals (lead and aluminium) on *Prochilodus lineatus* and *Astyanax bimaculatus*, two freshwater fish species found in a variety of polluted waterways in the south of Brazil. Fish health was assessed by some physiological parameters such as: hematocrit, sodium and glucose plasma concentrations.

## Material and Methods

Juveniles of *Prochilodus lineatus* and adults of *Astyanax bimaculatus* were obtained from the University Hatchery Station. After acclimation, short-term static toxicity tests (24h for Trifluralin and 96h for Azodrin and metals) were carried out. Animals were exposed to concentrations corresponding to 25 and 75% of the LC<sub>50</sub> (median lethal concentration). Experiments with metals were performed in an 80 L glass aquarium and with pesticides in a 140 L tank. Water was continuously aerated, temperature was kept at 21±1°C and pH 7.5. Only the experiments with aluminium were conducted in acidified water (pH 5,0). Blood samples were taken from caudal vein after 6, 12, 24, 48, 72 and 96 hours of exposure to pollutant and after 96 hours of exposure only to the dilution water (control group). Experiments with Trifluralin lasted only 24 hours and blood samples were taken after 6, 12 and 24h. After blood centrifugation, hematocrit was determined using a microhematocrit scale. Plasma sodium concentrations were measured by flame photometry and glucose concentrations by spectrophotometric enzymatic assay. Differences among groups exposed to the same pollutant concentration, for different time periods, and the control group were tested for significance by one way ANOVA and multiple range test (Student-Newman-Keuls). Means were considered different where P < 0.05.

## Results

As it is shown in Table 1 *P. lineatus* exposed to the highest concentrations of Azodrin, lead nitrate and aluminium sulfate showed significant reduction on plasma sodium concentrations and a return to sodium control levels after 72 hours. Fish exposed to both metals concentrations, as well as to 0.19 ppm of Trifluralin, showed significant increase on blood glucose that lasted longer in animals exposed to aluminium. Only animals exposed to the highest aluminium concentration showed a significant variation on hematocrit: an increase after 6 and 48 hours of exposure.

The results obtained for *Astyanax bimaculatus* are presented in Table 2. An initial decrease on blood sodium concentration, followed by a significant increase, was observed in fish exposed to both lead concentrations. Aluminium exposure during 24 and 48 hours led to a decrease on this parameter. Animals exposed to both Azodrin concentrations and to the lowest Trifluralin and lead concentrations showed a significant increase on blood glucose. On the other

hand, *A. bimaculatus* exposed to the highest concentrations of the herbicide and aluminium showed blood glucose reduction.

Table 1 - Significant increase (↑) or decrease (↓) on hematocrit, blood sodium and glucose concentrations of *P. lineatus* after the exposure to sublethal concentrations of Azodrin, Trifluralin, lead nitrate or aluminium sulfate, in relation to the control group.

Pollutant	Hematocrit	Sodium	Glucose
<b>Azodrin</b>			
7.0 ppm	-	-	-
21.0 ppm	-	↓ 6h ↑ 12 and 48h	-
<b>Trifluralin</b>			
0.06 ppm	-	-	-
0.19 ppm	-	-	↑ 6, 12 and 24 h
<b>Pb (NO<sub>3</sub>)<sub>2</sub></b>			
38.0 ppm	-	-	↑ 6, 12 and 24 h
114.0 ppm	-	↓ 48h	↑ 6 and 24 h
<b>Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub></b>			
0.1 ppm	-	-	↑ 6, 48 and 72 h
1.0 ppm	↑ 6 and 48h	↓ 6 and 24h	↑ from 6 to 96 h

### Discussion and Conclusion

The observed decrease in Na<sup>+</sup> plasma concentration in both fish species exposed to the insecticide and metals might be indicating a decrease in sodium influx rate in consequence of the inhibition of Na,K,ATPase in gills. The posterior return of sodium concentrations to control values suggests a recovery of osmoionic homeostasis, even with continued pollutant exposure, where cortisol might be playing a key role. The observed hyperglycemia in both species exposed to pesticides and lead reinforces this cortisol role. Blood glucose reduction in *A. bimaculatus* exposed to Trifluralin and aluminium might reflect glucose urinary loss, probably in response to renal reabsorption disruption. The hematocrit increase showed by both species exposed to aluminium might be rather related to the low pH than to the metal itself. In conclusion, the present study points out that sublethal concentrations of Azodrin, lead and aluminium interfere on sodium regulation and glucose metabolism of *P. lineatus* and *A. bimaculatus*

and these parameters might be useful tools in monitoring pesticides and metal effects.

Table 2 - Significant increase (↑) or decrease (↓) on hematocrit, blood sodium and glucose concentrations of *A. bimaculatus* after exposure to sublethal concentrations of Azodrin, Trifluralin, lead nitrate or aluminium sulfate, in relation to the control group.

Pollutant	Hematocrit	Sodium	Glucose
<b>Azodrin</b>			
253.0 ppm	-	-	↑ 6h
758.0 ppm	-	↑ 72h	↑ 12, 24 and 48h
<b>Trifluralin</b>			
0.30 ppm	-	-	↑ 12 and 24h
0.91 ppm	-	-	↓ 6, 12 and 24h
<b>Pb (NO<sub>3</sub>)<sub>2</sub></b>			
33.0 ppm	-	↓ 24h; ↑ 48h	↑ 12 and 24h
100.0 ppm	-	↓ 6h; ↑ 72 and 96h	-
<b>Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub></b>			
0.1 ppm	↑ 6h; ↓ 72h	↓ 24h and 48h	-
1.0 ppm	↑ 48 and 96h	↓ 24h	↓ 24 and 48h

- indicates no significant variation

### Acknowledgments

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**GILL CHLORIDE CELLS AND GAS TRANSFER  
IN FISH EXPOSED TO DEIONIZED WATER**

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**Abstract**

The effect of the proliferation of gill chloride cell on gas transfer was investigated in *Hoplias malabaricus*, exposed to deionized water for 1, 2 and 7 days. Chloride cell proliferation in the lamellar epithelium increased the water-blood diffusion barrier in all except fish kept in deionized water for 1 day. Oxygen consumption ( $\dot{V}O_2$ ) was significantly increased in fish kept in deionized water for 2 days, but all fish groups studied were able to keep  $\dot{V}O_2$  constant until the critical oxygen pressure ( $PcO_2$ ) was reached. No change in  $PcO_2$  was found in any groups. Respiratory frequency showed significant increase in fish kept for 2 days in deionized water while there was an increase in respiratory volume in fish kept 2 days in same water conditions. The arterial  $O_2$  partial pressure showed a significant decrease in normoxia and hypoxia in fish kept in deionized water. Plasma ions and pH were kept almost constant, although osmolarity decreased significantly in fish kept in deionized water for 7 days. These results confirm that the chloride cells in lamellar epithelium reduce the effectiveness of  $O_2$  uptake in *H. malabaricus*. However, other mechanisms should be involved in its capability to maintain the oxygen consumption up to  $PcO_2$ .

## **Introduction**

The fish gill is a multifunctional organ which serves in the passive exchange of oxygen, carbon dioxide, ions and nitrogenous metabolic products, and is also the site of active ion exchange and transport. As the gill is the main surface interface for the transfer of respiratory gases in most teleost fish, the effectiveness of gill structure depends on the lamellar surface, the distance of water-blood diffusion and the magnitudes and matching of gill ventilation and blood flow. However, the gill structure and its large surface area, which facilitates the respiratory gas movement, may give rise to problems in maintaining the ionic and osmotic steady state in fish body fluid.

The fish gill is organized in a sufficiently flexible way to allow fish to inhabit widely diverse and greatly varying environments (Laurent, 1989) and the gill epithelium generally responds to changes in the internal and external medium. Most studies have been addressed to respiratory or ion regulatory function and, over the last two decades, a number of researches have focused on the relationship between the gill morphology and the impairment of ionic homeostasis and its impact on the respiratory gas exchange (Perry, 1998).

Two epithelia are identified in the gill, i.e., the respiratory epithelium covering the lamella, which represents about 96 % of gill surface, and the non-respiratory epithelium covering the gill filament. Three cell types, pavement (PVC), chloride (CC) and mucous (MC) cells, are the main cells found in the outermost cell layer of gill epithelium. PVC is the most common cell type and is located on both filament and lamellar epithelia. MC and CC are dispersed in the leading and the trailing edges of filament or in the interlamellar regions. In some species, CC are found in the lamellae (Laurent, 1984). Morphological transformation of CC of euryhaline fish in response to transfer from freshwater to seawater and vice-versa has been well documented, as well as the CC proliferation on lamellar epithelium under conditions requiring an increase of ion transport capacity (Laurent 1989).

In seawater fish, the CC is the site of  $\text{Cl}^-$  and  $\text{Na}^+$  secretion; however, the role of CC is not well defined in the freshwater fish. Several studies have evidenced that CC in freshwater fish is the site of  $\text{Na}^+$ ,  $\text{Cl}^-$  (Avella et al, 1987; Perry et al., 1992 ) and  $\text{Ca}^{2+}$  (Flik e Verbost, 1993) uptake. Considering that the ionic composition of freshwater environments is extremely variable, ranging from

almost distilled water to water with high ion concentration, CC proliferation has been accepted as a common response of fish to ion poor water and, sometimes, high proliferation is found in the lamellar epithelium (Perry, 1998).

Contrary to PVC, the CC are round and large in diameter. Thus, increased numbers of CC on the lamella could potentially impair the gas transfer by modifying the diffusive conductance of the gill's surface due to the increased distance of the water-blood barrier diffusion (Bindon et al., 1994a, b; Fernandes et al, 1998). In this context, the purpose of this study was to evaluate the effect of gill CC proliferation on the oxygen transfer with emphasis on the oxygen uptake in normoxic and hypoxic conditions.

Brazilian freshwaters are generally ion poor and soft, with the exception of a few saline lakes on the coastal regions and the south of Brazil's pantanal (swampland) region. A preliminary analysis on the gills of species living in these environments showed great variability in the distribution, number and apical area of CC (unpublished data). Among these species *Hoplias malabaricus* has a large surface area (Fernandes et al., 1994) with an extremely thin respiratory epithelium consisting of PVC with rare chloride cells at the base of lamella (Fernandes e Moron, 1996). However, the exposure of *H. malabaricus* to water devoid of ion (extreme condition) stimulated CC proliferation (Moron, 2000).

### **Materials and Methods**

Adult *Hoplias malabaricus* weighing between 265 and 565 g were collected in the Mogi Guaçu basin, state of São Paulo, Brazil. The fish were kept in 1000 L tanks with continuous aerated flowing water (water composition:  $[Na^+] = 2.06 \pm 0.03$ ,  $[K^+] = 2.52 \pm 0.004$ ,  $[Ca^{2+}] = 4.00 \pm 0.013$ ,  $[Cl^-] = 0.5 \pm 0.04$ , pH = 7.0 ) at 25 °C and photoperiod 12L:12D, for at least 1 month before experiments. Fish were fed with live fish (small fish: *Astyanax sp*).

The fish were divided in two groups (n = 54 each); the control and the deionized water group. The control group was transferred to aquaria containing water with the same characteristics as those of the acclimation period, while the group 2 was transferred to aquaria with deionized water ( $[Na^+]$ ,  $[Ca^{2+}]$  and  $[Cl^-]$  no detectable). Two thirds of the water of each aquarium was renewed daily. One, two and seven days after their transference, fish randomly selected (n = 8) from each group were submitted to respirometry experiments in normoxia (partial

oxygen pressure-  $PO_2 = 140$  mmHg) and gradual hypoxia to determine the critical oxygen pressure ( $P_{cO_2}$ ). Oxygen consumption ( $VO_2 - mL_{O_2} kg^{-1} h^{-1}$ ) was determined using a flow-through respirometry system (Rantin et al., 1992). Concomitantly, the respiratory parameters (gill ventilation -  $V_G - mL_{H_2O} kg^{-1} min^{-1}$ , ventilatory volume -  $V_{S,R} - mL_{H_2O} kg^{-1} breath^{-1}$  and respiratory frequency -  $f_R - breath min^{-1}$ ) and oxygen extraction from water flow ( $EO_2 - \%$ ) were determined by measuring the inspired ( $PiO_2$ ) and expired ( $PeO_2$ ) water taken directly from the buccal and opercular cavities of fish via polyethylene tubes inserted in these cavities.

A second set of experiments was carried out after the  $P_{cO_2}$  was determined. The dorsal aortae of the fish was cannulated using a flexible polyethylene tube (PS 50), the experiments consisted of exposing fish to normoxic water following by gradual hypoxia until the  $PO_2$  reached  $P_{cO_2}$ , in which condition the fish were kept for 30 minutes. Samples of blood (500  $\mu$ L) were taken in normoxia and hypoxia to determine arterial blood  $PO_2$  ( $PaO_2$ ), make ion analyses ( $[Na^+]$ ,  $[K^+]$   $[Ca^{2+}]$  and  $[Cl^-]$ ) and determine pH.

After these experiments, the gills of each fish were immediately removed and fixed with 2.5% glutaraldehyde in 0,1 M phosphate buffer pH 7.4 at 4 °C and processed for analysis using light microscopy (LM) and scanning electron microscopy (SEM). Sections of 1  $\mu$ m thick were used to calculate the mean water-blood barrier distance according to the method described by Weibel and Knight (1964). The test-system to estimate distances in layered structures (Gundersen et al., 1988) were superimposed four times in each picture; an average of 100 measurements were made per fish. Cell surface parameters were analyzed using SEM. The apical CC area was determined according to Bindon et al. (1994b) by tracing the cell perimeter in a calibrated computer screen using a special software (Sigmascan, Jandel Scientific). In addition, the CC fractional area (CCFA), which represents the fraction of the epithelial surface of the gill filament occupied by CCs and cell density, was subsequently calculated using the following equations:

$$CCFA = \frac{\Sigma \text{ area of whole and partial CCs}}{10^6 \text{ picture area}}$$

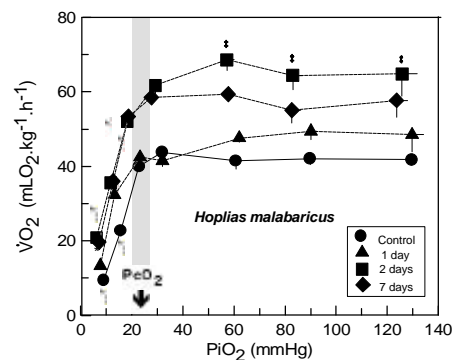
$$\text{Cell density} = \frac{CCFA}{\text{average whole cell area}}$$

The data were expressed as means  $\pm$  S.E.M. and the statistical significance of the differences between control fish and the fish kept in deionized water were determined using the GrapPad InStat v. 3.0 to Windows 95. The non-parametric

test Kruskal-Wallis ANOVA for multiple comparisons was used to compare the respirometric data followed by the Dunn Multiple Comparison test with 95 % confidence limits whenever a significance difference occurred. The hematological and CC morphometric data were based on parametric ANOVA tests and the Tukey-Kramer Multiple Comparison with 95 % confidence limits whenever a significance difference appeared.

## Results

Figure 1 shows the effect of the reduction of  $PiO_2$  on  $VO_2$  of control fish and on those kept in deionized water for 1, 2 and 7 days. The  $VO_2$  of fish kept for 48 h in deionized water was significantly greater than that of the controls in normóxia. Independently of experimental conditions,  $VO_2$  was kept constant during gradual hypoxia until it reached the  $PcO_2$ .

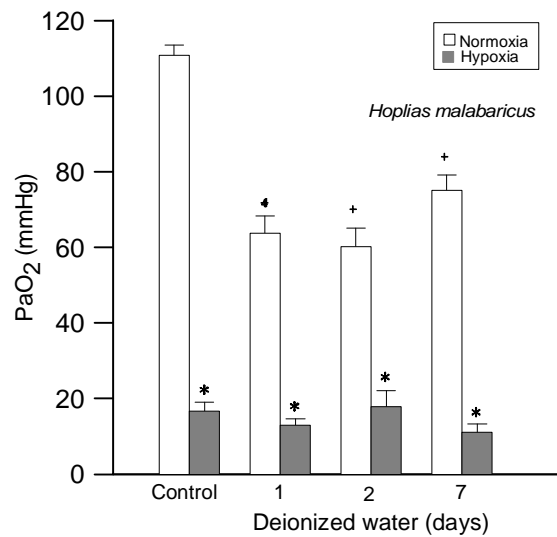


**Figure 1.** Effect of  $PiO_2$  on the  $VO_2$  in *H. malabaricus* from the control group and group maintained in deionized water 1, 2 and 7 days. The arrow indicates the mean  $PcO_2$ . Points are the means values  $\pm$  S.E.M,  $n = 8$ . (\*) indicates significant difference from normoxic data and (+) indicates significant difference from controls.

Below these  $PiO_2$  the  $VO_2$  became dependent upon the environment  $O_2$  availability. No significant difference was found in the  $PcO_2$  of either group and a mean  $PcO_2$  were calculated as  $PiO_2 = 21, 6 \pm 0,9$  mmHg. Gill ventilation ( $V_G$ )

showed no significant increase in normoxia and hypoxia although a significant increase was found in the  $f_R$  in fish kept 2 days in deionized water.

The effects of deionized water on  $PaO_2$  is shown in Fig. 2. The  $PaO_2$  of fish kept in deionized water was significantly lower than the control fish. Both the control group and the one kept in deionized water (1, 2 and 7 days) showed a significant decrease in  $PaO_2$  in hypoxia ( $PiO_2 = PcO_2$ ) compared with normoxic values.



**Figure 2.** Arterial oxygen partial pressure ( $PaO_2$ ) in controls and maintained in deionized water *H. malabaricus* in normoxia and hypoxia ( $PiO_2 = PcO_2$ ). + indicates significant difference ( $p < 0.05$ ) from controls in normoxia; \* indicates significant difference ( $p < 0.05$ ) between normoxic and hypoxic conditions in controls and deionized water fish.

Plasma  $[Na^+]$ ,  $[K^+]$ ,  $[Ca^{2+}]$  and  $[Cl^-]$  and blood pH were unaffected in fish kept in deionized water, however osmolarity decreased and was significant lower in fish maintained in deionized water during 2 and 7 days (Table I).

The distance of water-blood barrier on lamellae was smaller in fish kept 1 day in deionized water and greater in fish maintained 2 and 7 days in the same condition ( $p < 0.05$ )(Fig. 3). CC proliferated in the fish kept in deionized water and their apical surface area was significantly greater than that of control fish. Consequently, the fractional surface area of CC on the epithelia also increased ( $p < 0.05$ ).

**Table 1.** Plasma ion concentration, osmolality and pH in control and fish maintained in deionized water 1, 2 and 3 days. Values are means  $\pm$  1S.E.M.

	Control Group (n=24)	Deionized Water Group (n = 8 each)		
		1 Day	2 Day	7 Day
[Na <sup>+</sup> ] (mEq L <sup>-1</sup> )	128.06 $\pm$ 4.6	134.18 $\pm$ 4.2	128.29 $\pm$ 2.9	141.3 $\pm$ 6.6
[K <sup>+</sup> ] (mEq L <sup>-1</sup> )	3.7 $\pm$ 0.2	3.4 $\pm$ 0.4	3.2 $\pm$ 0.35	3.7 $\pm$ 0.35
[Ca <sup>2+</sup> ] (mEq L <sup>-1</sup> )	1.10 $\pm$ 0.04	1.18 $\pm$ 0.04	1.35 $\pm$ 0.07	1.13 $\pm$ 0.07
[Cl <sup>-</sup> ] (mEq L <sup>-1</sup> )	88.11 $\pm$ 6.0	97.8 $\pm$ 10.8	82.5 $\pm$ 9.8	82.9 $\pm$ 9.7
Osmolarity (mmol kg <sup>-1</sup> )	278.0 $\pm$ 15.5	255.0 $\pm$ 13.4	238.0 $\pm$ 5.6*	215.6 $\pm$ 11.6*
pH	7.8 $\pm$ 0.04	7.8 $\pm$ 0.07	7.9 $\pm$ 0.04	7.8 $\pm$ 0.02

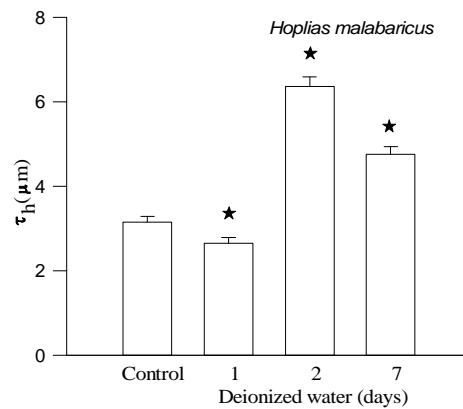
(\* ) indicates a significant difference from the control value,  $p < 0.05$ .

## Discussion

The rate of oxygen transfer across the lamellar epithelium is inversely related to the thickness of the water-blood diffusion barrier. The morphometric diffusion capacity given as  $KA/t$  where  $K$  is the Krogh permeation coefficient,  $A$  is the respiratory area and  $t$  is the water-blood barrier thickness has been estimated in several fish species (Hughes, 1984). Generally, fish gills with larger surface areas tend to have thinner barriers and consequently much greater diffusing capacities (Hughes, 1984). Thus, the enlargement of the distance of the water-blood diffusion barrier due to CC proliferation in the lamellar epithelium potentially cause an impairment of gas transfer. Early studies have shown a significant reduction of PaO<sub>2</sub> in normoxia and hypoxia (Thomas et al., 1988, Bindon et al. 1994b, Greco et al., 1995).

In the present study, *H. malabaricus* kept in deionized water exhibited two types of responses related to CC proliferation. Fish kept 1day in deionized water did

not increase the water-blood diffusion distance although the lamellar thickness increased due to the enlargement of blood spaces and the changes on CC were restricted to those cells in the filament epithelium. Fish kept in deionized water for 2 and 7 days exhibited high CC proliferation in lamellar epithelium and increased the water-blood diffusion barrier. All fish kept in deionized water showed a significant reduction in PaO<sub>2</sub>.



**Figure 3.** The effect of dionized water on the harmonic mean water-blood barrier distance ( $\tau_h$ ). Values are means  $\pm$  S.E.M. (\*) indicates significant difference from controls ( $p < 0.05$ )

However, *H. malabaricus*, whose VO<sub>2</sub> is low, showed be an oxyregulator species even under this extremely adverse condition. The PcO<sub>2</sub> values were similar to those calculated for this species by Rantin et al. (1992), denoting a marked ability to compensate for reduced environmental O<sub>2</sub> pressure. The maintenance of VO<sub>2</sub> implies the need for ventilatory adjustments in an attempt to keep EO<sub>2</sub> from water and, hence, the O<sub>2</sub> cascade from water to tissue. However, EO<sub>2</sub> generally decreases with very large ventilation volume which increases the branchial dead space. Increased f<sub>R</sub> such as was found in *H. malabaricus* could be a compensatory mechanism to counteract with the potential loss of gas transfer effectiveness associated with changes in the diffusion barrier, as suggested by Greco et al (1995) in their studies with rainbow trout, *Oncorhynchus mykiss* acclimated in softwater. Although, the increase in f<sub>R</sub> represents a higher increase in the metabolic cost of ventilation, *H.*

*malabaricus* has lower ventilatory cost than other species already studied (Rantin et al., 1992). Its large respiratory surface area (Fernandes et al., 1994), high O<sub>2</sub> affinity of hemoglobin (Wood and Lenfant, 1979) and high anaerobic capacity (Hochachka et al., 1978) may favor this species to maintain VO<sub>2</sub>.

The plasma ion levels of *H. malabaricus* kept in deionized water remained unchanged despite the significant reduction on plasma osmolarity. These findings are difficult to explain in fish kept in deionized water. Greco et al. (1995) found significant depression in plasma [Cl<sup>-</sup>] in *O. mykiss* acclimated to softwater. Thus, the proliferation of CC and the increase in its apical surface together with the possible activation of other regulatory mechanisms may maintain plasma ion levels in *H. malabaricus* kept in deionized water.

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**THE EFFECTS OF SIMULATED GLOBAL WARMING  
ON THE GROWTH AND ENERGETICS  
OF FRESHWATER FISH**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Fish are likely to be amongst those animals most affected by global warming as their metabolic rate and physiology are fundamentally affected by environmental temperature. However, little of the vast literature on temperature physiology in fish relates to a small temperature increment in the natural environment (Wood and McDonald, 1997). Therefore, in 1992, we began a series of experiments to study the effects of simulated global warming on the growth and energetics of juvenile rainbow trout (*Oncorhynchus mykiss*). We added 2°C to the *natural* thermal regime, in the presence and absence of two common freshwater pollutants in environmentally relevant media: ammonia in hard water (HW) and acidity (low pH) in soft water (SW). The experiments lasted 90 days in summer and in winter at a satiation ration ( $\approx 2.5\%$  wet body weight  $d^{-1}$ ) and in summer at a limited ration of  $1\% d^{-1}$ . The present paper summarizes the most important findings of the project.

The addition of 2°C had little effect on the growth and physiology of juvenile trout fed to satiation over most of the summer, suggesting that there is physiological compensation for the additional temperature. However, at high

ambient temperature in late summer, +2°C caused a marked inhibition of appetite and growth in both SW- and HW-acclimated fish (Table. 1). A reduction in ration in summer to 1% d<sup>-1</sup> did not increase the impact of simulated global warming. In winter, +2°C stimulated appetite and growth by approximately 30% in HW-acclimated fish and 60% in SW-acclimated fish.

Table 1. Effects of simulated global warming (+2°C) on appetite and growth of satiation-fed rainbow trout over the 30-day period of maximum (late) summer temperatures (20 – 24°C). Values are means ± SEM. An asterisk indicates a value at +2°C that is significantly different to that at ambient temperature ( $P < 0.05$ ).

	SW-acclimated fish		HW-acclimated fish	
	Ambient†	+2°C	Ambient	+2°C
Appetite (g d <sup>-1</sup> )	0.68 ± 0.08	0.49 ± 0.08*	0.66 ± 0.07	0.46 ± 0.07*
Growth rate (g d <sup>-1</sup> )	0.58 ± 0.04	0.07 ± 0.01*	0.72 ± 0.05	0.26 ± 0.03*
Conversion ratio	0.85 ± 0.07	0.14 ± 0.02*	1.10 ± 0.09	0.57 ± 0.07*

†Ambient temperature in SW was approx. 2-3°C greater than that in HW.

The high sensitivity of growth to the experimental treatments was consistent throughout the project. The most sensitive physiological indicator of +2°C was tissue protein metabolism i.e. rates of protein synthesis, accretion and degradation (Houlihan et al., 1995). For example, protein accretion in liver and white muscle was significantly (approx. 20%) reduced by +2°C in the late summer due to an increase in protein degradation.

Sublethal ammonia inhibits growth in freshwater fish (Thurston et al., 1984) and therefore we were very surprised to find that fish exposed to 70µM total ammonia ( $T_{\text{Amm}} = \text{NH}_3 + \text{NH}_4^+$ ) in summer were heavier, had greater N-absorption and retention efficiencies, and higher energy conversion efficiency. They also had higher plasma [ammonia], and greater liver and white muscle protein synthesis rates. We suggested that the increase in N-retention could be the indirect result of ammonia detoxification whereby ammonia was incorporated into glutamine and other amino acids, which could then be used as substrates for protein synthesis, but this idea remains highly speculative.

Low pH (5.2) exposure unexpectedly tended to stimulate appetite and growth but caused no disturbance of electrolyte balance, a typical symptom of acid toxicity (Morris et al., 1989). We hypothesized that the fish were able to replace branchial ion losses with dietary salt: when we further reduced ration to maintenance levels ( $1\% 4d^{-1}$ ), exposure to pH 5.2 indeed resulted in lower whole-body  $[Na^+]$  and  $[Cl^-]$  and mortalities of 34% compared to 10% at the control pH 6.2.

Sublethal toxicant exposure exacerbated the effects of  $+2^\circ C$ . For example, at the high ambient temperatures of late summer, ammonia in HW or low pH in SW increased the negative growth effects of  $+2^\circ C$ . Similarly, at a (winter) maintenance ration, exposure to both  $+2^\circ C$  and low pH caused a greater ionoregulatory disturbance than  $+2^\circ C$  or low pH alone. In satiation-fed fish in winter, those exposed to  $+2^\circ C$  and sublethal toxicants had the highest appetite, growth and food conversion efficiency.

The ability of the fish to acclimate to the experimental conditions was tested by challenging fish with acute lethal temperature and/or toxicant concentrations. Fish exposed to  $+2^\circ C$  had a slightly ( $0.2-1.0^\circ C$ ) but significantly higher lethal temperature than those exposed to ambient temperature when fed to satiation. However there was no evidence of acclimation to ammonia or low pH; pre-exposure to sublethal toxicants offered no protection to subsequent acute lethal challenges.

In conclusion, the impact of global warming on freshwater fish will vary seasonally. The additional temperature may provide growth benefits in winter, but may threaten fish populations living towards the upper end of their thermal tolerance zone in (late) summer. The project also demonstrated that the effects of pollutants under environmentally realistic conditions might be rather different from those in the classical, physiological literature.

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**RNA SYNTHESIS AND ANOXIC SURVIVAL**  
**IN CRUCIAN CARP (*CARASSIUS CARASSIUS*)**

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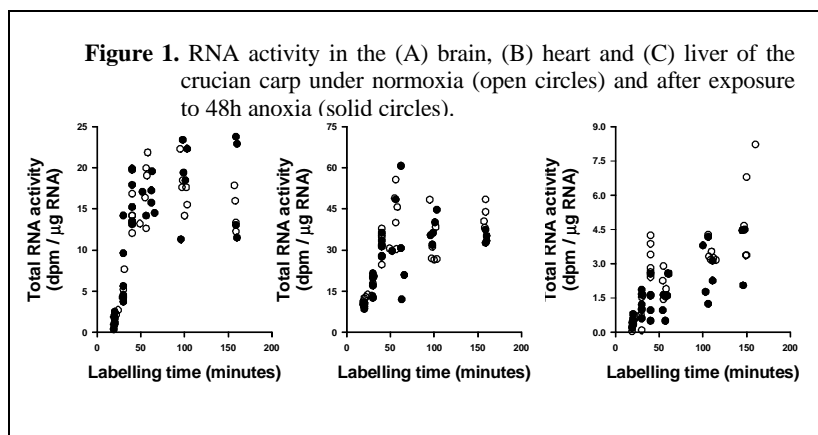
**EXTENDED ABSTRACT ONLY - DO NOT CITE**

During anoxia the crucian carp downregulates protein synthesis in the liver, heart and muscle, yet maintains synthesis in the brain. This results in a 40% reduction overall and ensures neuronal survival (Smith *et al*, 1996). Tissue specific downregulation is associated with reductions in RNA to protein ratio (heart) or RNA translational efficiency, *i.e.* mg protein synthesised  $\mu\text{g}^{-1}$  RNA day<sup>-1</sup> (liver, and muscle), whereas an increase in brain RNA translational efficiency counters the reduction in RNA to protein ratio (Smith *et al*, 1996). Since we know little about how events upstream of RNA translation affect anoxic protein synthesis, here we describe how changes in RNA synthesis contribute to anoxic survival.

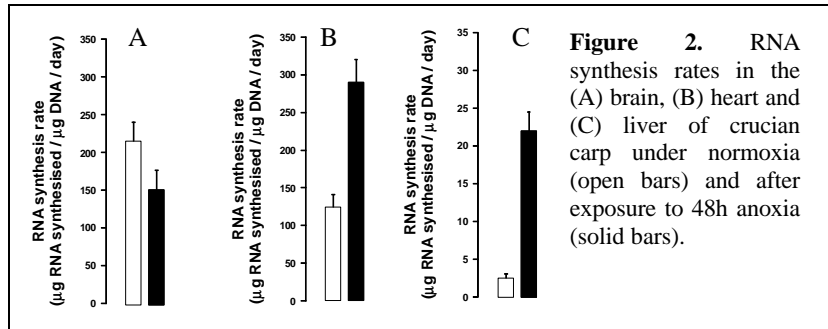
After normoxia or 48 h anoxia (described by Smith *et al*, 1996), each fish was injected with 10  $\mu\text{l}$   $\text{g}^{-1}$  body weight of 100 mM uridine @ 3.7 MBq (100  $\mu\text{Ci ml}^{-1}$ ) <sup>3</sup>H-uridine and returned to the appropriate conditions. Individuals were then killed and the brain, heart ventricle and liver removed and immediately frozen. Free intracellular nucleotides were liberated by homogenisation in 0.5M perchloric acid (PCA) and were analysed by HPLC, with the eluted UMP, UDP and UTP fractions being collected (Smith *et al*, 1999). PCA insoluble material was dissolved in NaOH and RNA extracted by precipitation of DNA and protein with 20% PCA. RNA (and also DNA, following further PCA extraction) was

determined by dual absorbance spectrophotometry. Radioactivity levels were determined by scintillation counting.

UDP was not detected in any tissue and UTP was not detected in the heart. Stabilised normoxic UMP specific activities ( $\text{dpm nmol}^{-1}$ ) were ranked: liver ( $490.2 \pm 63.2$ ) > heart ( $99.6 \pm 12.5$ ) > brain ( $43.1 \pm 6.3$ ). Under anoxia liver UMP specific activity declined whereas the brain and heart were unaffected ( $201.3 \pm 25.6$ ,  $36.7 \pm 3.2$  and  $81.7 \pm 8.6$   $\text{dpm nmol}^{-1}$ , respectively). Stabilised normoxic brain and liver UTP specific activities were similar ( $5.2 \pm 1.8$  and  $7.5 \pm 1.6$   $\text{dpm nmol}^{-1}$ , respectively). Both were increased by anoxia ( $11.9 \pm 3.1$  and  $13.3 \pm 2.8$   $\text{dpm nmol}^{-1}$ , respectively). RNA radioactivities ( $\text{dpm } \mu\text{g}^{-1}$ ) increased linearly, with labelling time, for 40 mins. Afterwards there were no further changes (Fig. 1).



From the linear RNA labelling phase and total nucleotide specific activities we have developed a novel RNA synthesis rate calculation (Smith *et al*, 1999). This allows for the differential uridine salvage, with synthesis rates expressed relative to DNA (Fig. 2). This defines tissue specific RNA synthesis rates, brain > liver and show that, after 48 h anoxia, RNA synthesis is reduced in the brain yet is increased in the heart and the liver (Fig. 2).



RNA synthesis is not downregulated during anoxia. However these changes in synthesis rate do correspond to known anoxic survival mechanisms. In the brain these data confirm an earlier investigation where neuron survival is increased following RNA synthesis inhibition; RNA synthesis being required for neuron apoptosis (Rosenbaum *et al*, 1994). In the liver, a reduction in newly synthesised proteins reduces rRNA survival yet enhanced recovery depends on the replacement of pre-existing ribosomes plus the synthesis of specific mRNA's, as shown in anoxia tolerant turtles (Douglas *et al*, 1994). Thus, the large reduction in liver protein synthesis rate (>95%; Smith *et al*, 1996) corresponded to the greatest increase in RNA synthesis rate (>800%; Smith *et al*, 1999).

These data provide evidence that these survival strategies are possible because they conform to the combined energetics of RNA and protein synthesis; *i.e.* contains both a variable and fixed component, the latter being RNA synthesis (refer to Smith and Houlihan, 1995). Nucleotide supply constitutes the greatest energy demand of RNA synthesis, but exogenous salvage avoids the higher costs of intracellular synthesis (Rudolph, 1994). Although the extent of nucleotide salvage (Smith *et al*, 1999) corresponded to normoxic protein synthesis rates (Smith *et al*, 1996) there was no increase of exogenous salvage (as could be expected) under anoxia. However in the liver an increase in RNA species, with an increased turnover, could, via nucleotide recycling, reduce even the necessity for exogenous salvage. It would therefore be of interest to know exactly which nucleotide sequences are being transcribed. In the brain, a reduction in RNA synthesis would incur an energetic commitment to intracellular synthesis in order to maintain a fixed RNA synthesis cost.

RNA synthesis may therefore represent an area where energy demand is not so much downregulated as restructured. Therefore maintaining the fixed cost of RNA synthesis may be as vital to anoxic survival as exploiting the variable cost of protein synthesis (Smith *et al*, 1999).

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**REDUCED GROWTH OF ATLANTIC COD (*GADUS MORHUA*)  
IN CHRONIC HYPOXIA:  
THE EFFECT OF VARYING MEAL FREQUENCY**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

In the Gulf of St. Lawrence, Atlantic cod frequently spend time in waters with low levels of dissolved oxygen (hypoxia). In a previous experiment (Chabot and Dutil 1999), we showed that growth rate of Atlantic cod declined when dissolved oxygen fell below 73% saturation. At 45% saturation, growth in length and mass was reduced by 35 and 56%, respectively, relative to growth in normoxic water. Hypoxia-induced reductions in growth rate were due to decreased ingestion rates. It is not clear if a post-prandial peak in oxygen consumption limits food intake in hypoxia, or if cod digest meals at a slower rate, and as a result reduce their food intake. If the former was true, cod should be able to increase food intake in hypoxia by taking more frequent, smaller meals. To verify this hypothesis, we compared growth in cod exposed to 3 different meal frequencies, in hypoxia and normoxia.

**Methods**

We varied meal frequency (1, 3, and 7 meals per week) for cod held at two regimes of dissolved oxygen (40 and 90% saturation). Two replicate tanks with 9 or 10 fish each were used for each combination of oxygen level and meal frequency. For each meal, cod were fed capelin ad lib for one hour. Temperature and salinity were held constant (10°C and 28‰). Fish were tagged to allow

determination of individual growth rates in mass (g/d and specific growth,  $100 \times (\ln(\text{final mass}) - \ln(\text{initial mass}))/56$  days), length (mm/d) and condition (Fulton K) at the end of the experiment. In addition, food consumption was measured for each tank.

## Results and Discussion

In general, growth increased with feeding frequency in normoxia, but was little affected by feeding frequency in hypoxia (Figure 1). We noted marked differences between replicates at 3 meals per week, for both oxygen levels. The reasons for this are not clear, but could be related to small sample size in each

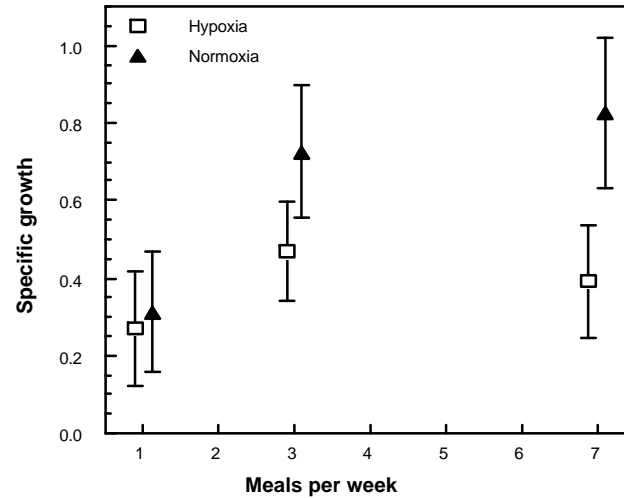


Figure 1. Growth rate of cod according to oxygen level and meal frequency (mean of fish in two tanks, with 95% CI).

tank, and to differences in initial size of fish between tanks, despite random allocation of the fish. However, a 2-way Anova with replicates as a nested factor showed that overall, replicates for each treatment did not differ significantly ( $F_{[6,102]}=1.03$ ,  $p=0.4$ ). Replicates were therefore pooled to increase the power of the Anova.

The interaction was significant ( $F_{[2,108]}=3.48$ ,  $p=0.034$ ), indicating that changes in meal frequency did not affect growth in the same manner for both oxygen levels. Multiple comparisons of means showed that in normoxia, growth was significantly reduced at 1 meal per week compared to 3 and 7 meals. In hypoxia, however, there was no difference in growth between the 3 feeding frequencies. At 1 meal per week, growth was equally poor at both oxygen levels. At 3 and 7 meals per week, growth was faster in normoxia than in hypoxia, although this was significant only at the highest feeding frequency. In our previous experiment (Chabot and Dutil 1999), growth was significantly greater in normoxia than at 45% oxygen saturation, at a feeding frequency of 3 meals per week. Possibly the larger range of fish sizes and the lower density of fish in each tank allowed aggressive interactions which increased variability in the present experiment.

The same conclusions were reached when growth in length and changes in condition were compared for the 2 levels of dissolved oxygen and the 3 meal frequencies.

As in Chabot & Dutil (1999), differences in growth between the different treatments were entirely mediated by differences in food ingestion (Figure 2), and a single relationship fitted all the data, irrelevant of whether reductions in food ingestion were caused by hypoxia or feeding frequency.

Furthermore, this relationship was almost identical to the one reported by Chabot and Dutil (1999). The slope was slightly but significantly lower in the latter (0.285 vs 0.347,  $F_{[1,14]}=8.02$ ,  $p=0.013$ ), but this difference disappears after removing the surprisingly low growth observed at 93%  $O_2$  saturation in 1995. Excluding this point and pooling data from both experiments leads to the following relationship:

Growth rate (in g/d) =  $-1.398 + 0.338 \cdot$  ingestion rate (in g/d),  $r^2 = 0.99$   
Assimilation efficiency varied between treatments, but was very highly correlated with food ingestion, and had little impact on growth rate compared with food ingestion.

It appears that hypoxia and low feeding frequencies result in lower food intake, which in turn reduces growth rate. Our results also suggest that cod digest food so slowly in hypoxia that growth rates are the same whether they have access to food 1, 3 or 7 times per week. Cod cannot reduce the negative impact of hypoxia on growth by taking more frequent meals.

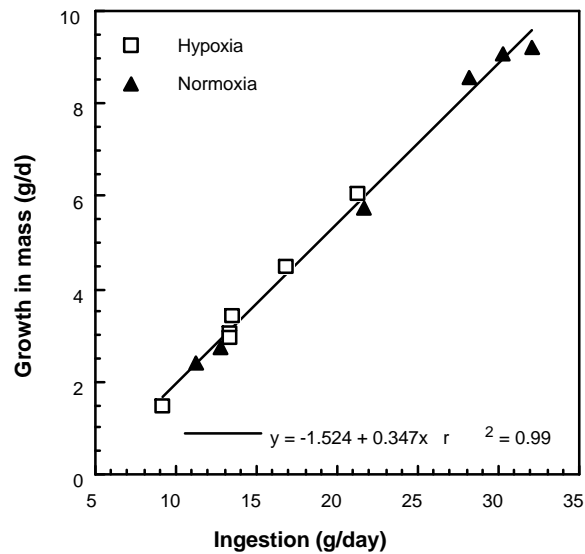


Figure 2. Relationship between growth and food ingestion.

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**USE OF SURFACE RESPIRATION BY**  
***FUNDULUS HETEROCLITUS* AND *FUNDULUS MAJALIS***  
**FROM HYPOXIA-PRONE WESTERN LONG ISLAND SOUND**

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**Abstract**

Cyprinodonts such as the mummichog, *Fundulus heteroclitus*, and the striped killifish, *Fundulus majalis*, are abundant in stressed environments such as the bays and harbors of western Long Island Sound, which frequently become hypoxic during the summer months. Use of surface respiration during periods of environmental hypoxia could increase their tolerance to low dissolved oxygen (DO). We found that both species of killifish altered their respiratory behavior when exposed to progressive hypoxia in the laboratory and adopted aquatic surface respiration increasingly after oxygen concentrations declined below 5.0 mg O<sub>2</sub>/l. In addition, below 4.0 mg O<sub>2</sub>/l, both species were observed to break the water surface, apparently to take air into the buccal cavity. Striped killifish reached maximal opercular rate, and initiated the use of surface respiration, at higher dissolved oxygen concentrations (DO) than mummichogs. The frequency of surface respiration in striped killifish was at least twice that of mummichogs at dissolved oxygen concentrations from 5.0 to 2.0 mg O<sub>2</sub>/l. In severe hypoxia of 1.0 mg O<sub>2</sub>/l both species spent considerable time (>80%) at the surface using these alternate modes of respiration (ASR and air gulping).

**Introduction**

The Cyprinodonts are abundant in bays, harbors, and marshes along the East Coast of the North America (Bigelow & Schroeder, 1953; Diaber, 1982; Kneib, 1986; McEnroe et al., 1994) where they are import forage species for

piscivorous fishes and wading birds (Frederick and Loftus, 1993). Both mummichogs, *Fundulus heteroclitus*, and the striped killifish, *Fundulus majalis*, are estuarine species (Nixon & Oviatt, 1973; Kneib & Stiven, 1978). Although these congeners are sympatric, *F. heteroclitus* is common in both subtidal and intertidal areas of the marsh while *F. majalis* occurs primarily in subtidal areas of the marsh (Weisberg, 1986).

Long Island Sound is heavily impacted by anthropogenic inputs and the deeper waters of the western Sound usually become hypoxic during the summer (LISS, 1994). Near shore areas may also become hypoxic (Parker, 1991). We have found a diurnal variation in oxygen at our study site, with oxygen minima in the early morning and maxima in the afternoon (McEnroe, unpubl.). While both species of *Fundulus* are common in near shore areas of the Sound, field studies found *F. heteroclitus* occurs in high numbers, even at low dissolved oxygen levels while the abundance of striped killifish, *F. majalis*, is more variable (McEnroe et al., 1994).

Among fishes there is a wide array of physiological, morphological, and behavioral adaptations to hypoxia, including aquatic surface respiration (ASR), bimodal respiration and air breathing. Even in hypoxic water the air-water interface is relatively well oxygenated and the use of this water layer for respiration is termed aquatic surface respiration (Kramer, 1982). Such alternative modes of respiration are well documented for tropical freshwater fishes from hypoxia-prone habitats. However, fewer studies have been made on temperate estuarine species. In such habitats hypoxia is linked to anthropogenic inputs and presumably is of recent origin.

Surface respiration may permit fish to survive otherwise lethal oxygen levels (Kramer & McClure, 1981a) by increasing arterial oxygen content (Burggren, 1982). ASR is performed by fish moving to the surface of the water column and ventilating their gills with the top 1 cm of the water (Lewis, 1970; Gee et al, 1978; Kramer, 1982, 1983). Although energetically expensive (Kramer & McClure, 1981a) the cost of ASR may be reduced by use of a buccal bubble to enhance buoyancy (Gee, 1991; Gee & Gee, 1991). Killifishes (*Fundulus* spp.) have several morphological characteristics important in the use of surface respiration: a dorsally oriented mouth, neutral buoyancy, and small body size (Lewis, 1970). We investigated the respiratory responses, including use of surface respiration (ASR and air gulping), of *F. heteroclitus* and *F. majalis* to low dissolved oxygen.

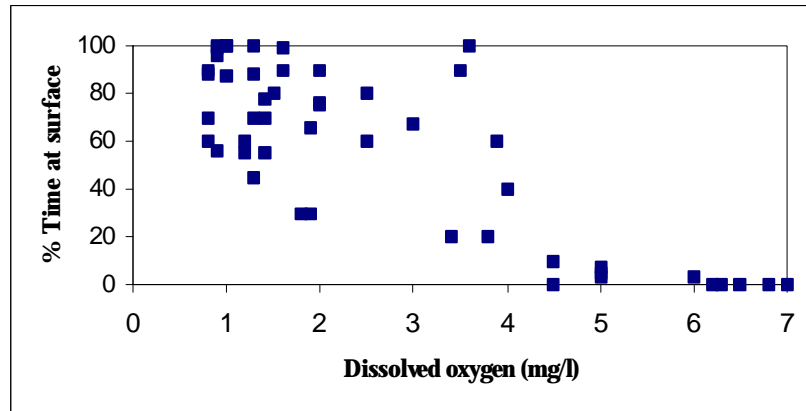
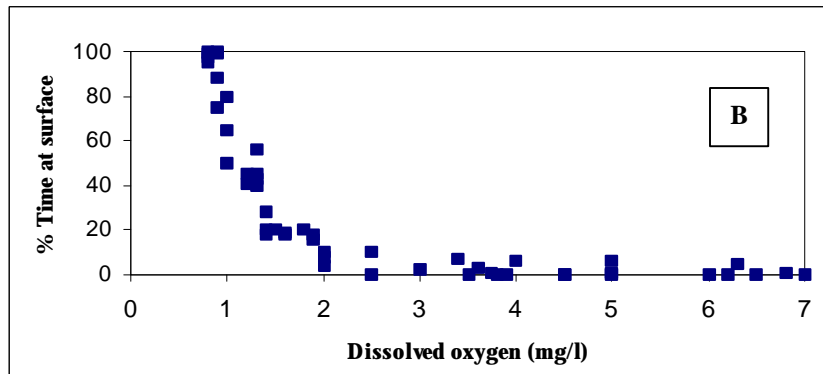


Figure 1. Time spent at the surface by *Fundulus majalis* (A) *Fundulus heteroclitus* (B) exposed to progressive hypoxia. Each symbol represents the mean value for 5 fish at each oxygen level.



## Materials and Methods

Fish were collected by seine from in the western Sound in Rye NY. After transport to the laboratory they were held in aquaria in Long Island Sound water, at temperatures of 22-25 °C and salinity of about 26 ppt, typical of summer conditions in the Sound. Fish were fed Tetramin several times /d. The aquaria were fitted with undergravel filters and aerated to maintain dissolved oxygen concentrations at 6-7 mg O<sub>2</sub>/l. Water was renewed periodically.

Fifteen experiments were conducted, five on *F. majalis*, and ten on *F. heteroclitus*. For each, 5 fish were placed in a 25 liter glass experimental tank and acclimated for several days prior to experiments. Fish were fed several hours prior to the start of the experiment and left over food removed from the tank. Dissolved oxygen concentration (DO, mg O<sub>2</sub>/l) and temperature were measured using a YSI Model 58B Dissolved Oxygen meter fitted with a water stirrer, salinity was measured using a Riechert-Jung refractometer and control measurements obtained in normoxia. Then the dissolved oxygen (DO) concentration was lowered by stopping aeration and bubbling nitrogen (N<sub>2</sub>) into the tanks. In all experiments DO was continuously recorded and N<sub>2</sub> flow adjusted to decrease dissolved oxygen levels at a steady rate of about 1 mg O<sub>2</sub>/l/hr. Experiments lasted for 7 to 8 hrs, similar in length to the period of diel hypoxia in the marsh.

At each oxygen level, from normoxia (90-100% Saturation) to 1.0 mg O<sub>2</sub>/l, four measurements of respiratory behavior were made:

- a) opercular ventilation movements were counted for 15 sec on each fish.
- b) respiratory behavior was considered ASR when fish ventilated their gills at, or immediately below, the surface of the water. In each experiment, the total number of ASR events for 5 fish was counted during each 10 min. observation period.
- c) at low dissolved oxygen levels fish broke the surface with open mouths. This behavior was termed 'air gulping'; these events were quite distinct and easily differentiated from gill ventilation at the surface (ASR events). The total number of times the 5 fish in each experiment exhibited 'air-gulping' during each 10 min. observation period was counted.

d) percent time that all 5 fish in a tank spent at the surface was monitored over a 3 min period at each level of dissolved oxygen.

During each experiment the respiratory behaviors (a, b, c, d above) of the fish were measured at each of several levels of oxygen depletion, down to 1.0 mg O<sub>2</sub>/l.

## Results

### *Opercular Ventilation*

Measurement of opercular ventilation rate was made over a range of dissolved oxygen from 7.5 to 1.0 mg O<sub>2</sub>/l. Maximal opercular rates were similar, 170 bpm (*F. majalis*) and 175 bpm (*F. heteroclitus*), but *F. majalis* reached maximal opercular rate at 3.0 mg O<sub>2</sub>/l versus 1.0 mg O<sub>2</sub>/l in *F. heteroclitus*.

### *Percent Time at the Surface*

As the severity of hypoxia increased so did the use of ASR, thus fish spent more time near the surface of the water column (Figure 1a,b). In normoxia, 7.0 mg O<sub>2</sub>/l, both species spent less than 10% of the time at the surface of the water column. This increased to 93% and 87% respectively at 1.0 mg O<sub>2</sub>/l. However, striped killifish spent more time at the surface at intermediate oxygen concentrations of 6.0 to 3.0 mg O<sub>2</sub>/l.

### *Aquatic Surface Respiration*

Aquatic surface respiration was rarely observed at DO > 5 mg O<sub>2</sub>/l but as oxygen declined both species increased the frequency of ASR, exploiting the oxygen-rich surface layer of water to ventilate the gills. *F. majalis* initiated the use of ASR at a higher DO than did *F. heteroclitus*. The frequency of ASR increased in both species as oxygen levels fell but remained significantly greater in *F. majalis* than in *F. heteroclitus* until severe hypoxia of ≤ 1.5 mg O<sub>2</sub>/l (Figure 2). The percent of fish using ASR also increased with declining DO; ASR<sub>100</sub> was 2.0 mg O<sub>2</sub>/l in *F. majalis* and 1.0 mg O<sub>2</sub>/l in *F. heteroclitus*. Both species were observed to break the water surface, apparently to take air into the buccal cavity, as oxygen levels declined. This behavior was termed 'air gulping'; *F. majalis*

initiated this behavior at a higher DO (3.0 mg O<sub>2</sub>/l) than did *F. heteroclitus* (1.2 mg O<sub>2</sub>/l).

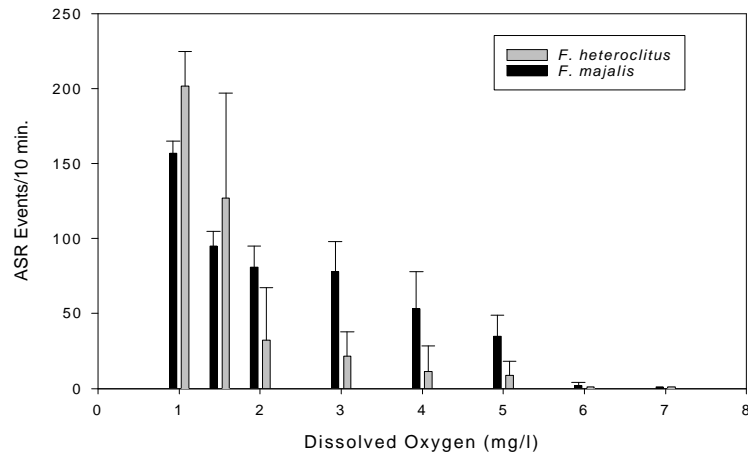


Figure 2. Use of aquatic surface respiration (ASR) by *Fundulus heteroclitus* and *F. majalis* exposed to progressive hypoxia. The mean  $\pm$  standard deviation is plotted for each of dissolved oxygen (DO) concentration. For both species the frequency of ASR was significantly higher (t-test,  $p < 0.005$ ) in hypoxia ( $DO \leq 3.0$  mg O<sub>2</sub>/l) than in normoxia ( $DO \geq 6.0$  mg O<sub>2</sub>/l). At intermediate oxygen levels of 3.0 to 5.0 mg O<sub>2</sub>/l, *F. majalis* used ASR more frequently than did *Fundulus heteroclitus*.

### Survival

All fish survived exposures of several hours at 1.0 mg O<sub>2</sub>/l were without apparent ill effects, the fish maintained their equilibrium and swam normally. At very low DO of 1.0 mg O<sub>2</sub>/l both species appeared stressed: *F. heteroclitus* changed color, and decreased swimming activity; *F. majalis* swam in an agitated manner.

## Discussion

In western Long Island Sound hypoxia occurs every summer, and in the harbors and embayments dissolved oxygen concentrations fluctuate with the diel cycle, reaching minima late in the night. Our measurements have confirmed that this cycle occurs nightly in Milton Harbor, Rye NY: the early morning measurements of oxygen approaching 3 mgO<sub>2</sub>/l (McEnroe et al., 1994). Mummichogs were abundant in this habitat, and remain there during hypoxic events (McEnroe et al., 1994). Such levels of hypoxia have been shown to produce physiological affects in marine fishes (Jensen et al., 1993). Hypoxia normally induces an increase in ventilation frequency (Burggren, 1982; McEnroe and Krozlowitz, 1997; Jensen et al., 1993) and stroke volume (Shelton, 1970; Jensen et al., 1993). Increasing gill ventilation passes more water over the gills per unit time, and can compensate for the decreased oxygen content of the water. However, there is a maximum ventilatory rate that can be achieved; as opercular rate increases, so does the amount of oxygen required to sustain it. Ventilatory costs in fish range from 0.5 to 43 % of the total oxygen consumption (Roberts, 1975; Holeton, 1980), with an average cost in normoxia of about 10% (Hughes, 1973). With increasing severity of hypoxia the cost of ventilation increases, and may eventually exceed the amount of oxygen taken up the gills (Burggren, 1982; Hughes, 1973). When oxygen becomes limiting opercular rate may decline (Fritsche and Nilsson, 1993; McEnroe and Krozlowitz, 1998). As expected, both killifish species increased ventilation rate in direct response to declining oxygen concentrations, and maximal opercular rates were similar in both species: 175 bpm in *F. heteroclitus* and 170 bpm in *F. majalis*. In both species ASR increased as opercular rates reached maximal values. Although no decline in opercular rate occurred in these experiments where fish had surface access, subsequent experiments (McEnroe et al., in prep.) have found such a decline when fish are denied access to the surface, suggesting that ASR enhances oxygen uptake and hypoxia tolerance.

Both species spent significantly more time at the surface in hypoxia than in normoxia. Percent time at the surface increased as oxygen declined: above 5.0 mg O<sub>2</sub>/l both species spent less than 10% of the time at the surface, and at 1.0 mg O<sub>2</sub>/l, both species spent most of the time at the surface (>80%). *F. majalis* initiated the use of ASR at a higher oxygen level than did *F. heteroclitus*, and spent proportionately more time at the surface. (>50%) than did *F. heteroclitus* (<30%) at intermediate oxygen levels (5.0 to 2.0 mg O<sub>2</sub>/l). The percent time spent at the surface correlated with the number of surface respiration events.

*Fundulus* are relatively small and both species of killifish studied possess dorsally oriented mouths and flattened heads that enable them to use the oxygen-rich surface layer of the water without altering swimming posture (Lewis, 1970). Furthermore, they are neutrally buoyant, an important feature for utilization of surface respiration. At oxygen levels below 5 mg O<sub>2</sub>/l the *F. heteroclitus* adopt ASR, swimming just beneath the air-water interface and utilizing the relatively oxygen-rich surface water to ventilate their gills. ASR was infrequent at dissolved oxygen concentrations above 5 mg O<sub>2</sub>/l, and the frequency of ASR increased dramatically as oxygen levels fell with ASR<sub>20</sub> reached at 3.2, and ASR<sub>100</sub> at 1.0 mg O<sub>2</sub>/l. Striped killifish (*F. majalis*) initiated the use of ASR at higher oxygen levels and used ASR more frequently than mummichogs when DO ≥ 1.5 mg O<sub>2</sub>/l. Both species were observed to break the water surface, apparently to take in an air, as oxygen levels declined. Air bubbles held in the buccal cavity may be used to oxygenate water flowing past it, thereby enhancing oxygen uptake at the gills. Burggren (1982) found goldfish using such a technique, had significantly higher arterial oxygen saturation than did fish denied surface access. Increased buoyancy from such bubbles could reduce the metabolic cost of surface respiration and enhance the efficiency of ASR (Gee and Gee, 1991).

The absence of surface respiration in normoxia suggests that such behavior has a cost, either energetic or in terms of aerial predation (Kramer, 1983; 1987; Kramer and McClure, 1981). Frequent trips to the surface, even in oxygenated water, uses a large percentage of the daily energy budget (Kramer and McClure, 1981; Pandian and Vivekanandan, 1976). The energetic cost of surface respiration may differ between the two *Fundulus* species: *F. heteroclitus* intermittently swam to the surface to perform surface respiration, while *F. majalis* stayed near the surface and constantly swam along the top of the water column. Chapman et al. (1995) suggested that rapid, constant swimming at the surface might force more well-oxygenated water over the gill than could be achieved by opercular ventilation alone, thus reducing the energetic cost of ASR. A second potential cost of surface respiration is increased risk of aerial predation. Cyprinodonts are important forage species for wading birds (Frederick and Loftus, 1993) and use of surface respiration has been found to increase predation risk for other species of fish (Kramer, 1983; 1987; Kramer and McClure, 1981). Although they were frequently collected together at low tide (McEnroe et al., 1994), *F. heteroclitus* inhabits both intertidal and subtidal areas while *F. majalis* occurs primarily in the subtidal areas (Weisberg, 1986). In the laboratory *F. majalis* appeared more stressed by hypoxia than its

congener: maximal opercular rate was reached at a higher dissolved oxygen than in *F. heteroclitus* (3.0 vs. 1.0 mg O<sub>2</sub>/l), and *F. majalis* initiated the use of ASR and air-gulping at higher DO than *F. heteroclitus*.

### Conclusions

Both species of killifish utilizes ASR and 'air-gulping' in hypoxia, as do many tropical fishes. In both, the frequency of surface respiration increased as O<sub>2</sub> declined. *F. majalis* initiated the use of surface respiration at higher O<sub>2</sub> concentrations than *F. heteroclitus*. In both species the use of surface respiration significantly increased when maximal opercular rate was reached, suggesting that these alternative modes of respiration were used to supplement oxygen uptake once gill ventilation could no longer suffice. We have found that other common species of fish from the Sound may use adaptive behaviors when exposed to hypoxia (McEnroe and Krozlowitz, 1997), though not all respond in the same way. Conventional laboratory assays measuring LC<sub>50</sub>s, or oxygen consumption, in response to acute exposures do not usually take account of adaptive responses therefore they may provide an incomplete measure of tolerance to hypoxia.

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GROWTH AND SURVIVAL OF JUVENILES OF TAMBAQUI  
EXPOSED TO DIFFERENT PHOTOPERIODS

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

The Amazon basin is the largest fluvial system of the world, draining about 37% of continental South America. The flood pulse is the main factor that influences the central Amazon areas characterized by a great variation in the water level and changes in ecological and physico-chemical parameters, such as pH of water, transparency, photoperiod, ion levels and dissolved oxygen. In the Amazon area we can find extremely different conditions among its three main types of waters: white waters (pH 6,2-7,2), black waters (pH 3,8-4,9) and clear waters (pH 4,5-7,8), according to Sioli (1984).

Tambaqui is widely distributed across South America. Tambaqui reaches a weight of more than 30 kg in wild. Its feeding habit (juvenile phase) ranges from the consumption of seeds and fruits during the rainy season to zooplankton and wild rice during the dry season. The flooded forest is the major representative habitat of tambaqui in the Amazon Basin, and this species occur in all types of Amazon waters.

The responses of fish to changing photoperiod include changes of rhythms of feeding and growth. Alterations of photoperiod are used to stimulate or delay gonadal maturation, and thus changing spawning period or somatic growth (decreasing energy drain for reproduction) (Lam, 1983). For example, Atlantic salmon (*Salmo solar*) show an increased growth when exposed to extended day length, but another salmonid, the Arctic charr (*Salvelinus alpinus*), may feed and grow well even in complete darkness (Jorgensen and Jobling, 1989). Silver catfish (*Rhandia quelen*) achieved better growth when exposed to continuous darkness (Piaia *et al.*, 1999).

The present communication describes the effect of three different photoperiods (control 10:14h light:dark, darkness 24h dark and light, 24h light) on growth and survival of juveniles of *Colossoma macropomum*.

The fish were distributed among six aquaria of 500l with 10 fish per aquarium (two replicates per treatment). The fish were exposed to continuous light, normal photoperiod (10h L and 14h D), and darkness (24h D) for 42 days. The aquaria water was maintained at 25 ( $\pm$ 1) $^{\circ}$ C and continuously aerated by 40W air pumps which promoted water circulation through a plastic mesh and stones to reduce water turbidity.

Fish were fed *ad libitum* once a day on ground commercial dry pellets (Purina 26% protein, digestible energy 2700 kcal/kg, water content of 13%, according to the manufacturer's instructions). Data are expressed as means (SE) and were analyzed by one-way ANOVA.

## **Results and Discussion**

The effect of photoperiod on the growth of fishes is probably related to the feeding and social habitats. Fish with nocturnal feeding habit, like catfishes, or those species that are aggressive, may increase food intake when held in darkness, whereas those with a diurnal habit may grow better on extended photoperiods. African catfish larvae (Britz & Pienaar, 1992) and Channel catfish (*Ictalurus punctatus*) showed better growth rates when exposed to continuous darkness. The Atlantic salmon (*Salmo solar*) show increased growth when exposed to extended day length (Berg *et al.*, 1992).

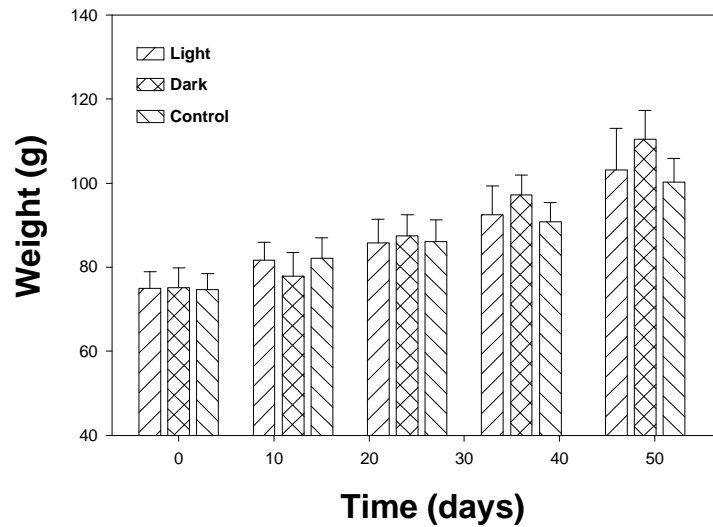


Figure 1. Effect of photoperiod on weight of *Colossoma macropomum* exposed to continuous light (24hs light), normal photoperiod (10:14 light:dark) and darkness (24hs dark). Values indicated are means (SE) of two replicates.

There was no mortality during the experiment with *Colossoma macropomum*. Weight of the fish reared in darkness were similar to those exposed to continuous light (Figure 1). Tambaqui is not usually described as an aggressive species. In wild, tambaqui experiences different conditions in water transparency. During high river water levels the animals can be found in the flooded forest where sunlight hardly reaches water surface and so the animals are exposed to dark. The animals living in várzea lakes where macrophytes cause extensive shadow face similar condition. This situation differs from that observed during low river water levels. However, the data presented here indicate that there is no significant change in growth rate of juveniles of tambaqui living in these different conditions.

### **Acknowledgements**

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**ABUNDANCE AND DISTRIBUTION OF FISH  
IN LITTER BANKS OF THE AMAZON**

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**Introduction**

The black water rivers from Amazonia originate from the lower and plain zones of the Guiana and Central Brazil shields, where erosion processes are not very intense and also reduced by the dense gallery forests. These rivers carry large amounts of humic and fulvic acids and are extremely poor in nutrients, due to water infiltration in the soil, which prevents a complete litter decomposition. There is almost no primary production in these environments and adjacent forests are the main source of nutrients. In small streams these resources concentrate in meanders, originating large litter banks to which several organisms associate. The trophic chain in this habitat starts with decomposing fungi, ending with small fishes and shrimps. Besides being a source of nutrients, litter banks also provide shelter against predators. These habitats are in a continuous process of decomposition and respiration.

**Material and Methods**

Data was collected in a small tributary (Tarumã-Mirim) of Negro River, 25 km from Manaus, during September, October and December 1999, which represent the dry season in the region. Three kilometers of the stream were mapped and

divided in 30 plots of 100 m. Ten plots were randomly selected to be sampled each month. Fishes were captured using a hand net in 10 throws per bank. Oxygen concentration was measured in a depth of 15 cm with an YSI MODEL 58 oxymeter. Total bank depth was measured with a graduated stack to the contact with the ground substrate. Bank volume was calculated by the product of length, width and depth. Flow speed in each bank was determined by the time necessary for a semi-floating body to cover 1m of distance.

### Results

We captured 3943 individuals, representing 6 orders, 17 families and 33 species during the period of study (Table 1). Oxygen concentration was highly correlated to bank volume and depth (statistics), but flow speed was not correlated to any of these variables. The abundance of each of the commonest species was not related to oxygen concentration and flow speed. Total number of species was negatively related to oxygen concentration ( $r^2=0.232$ ,  $p=0.000$ ) and flow speed ( $r^2=0.038$ ,  $p=0.000$ ), Figure 1.

Table 1. Number of animals captured, in brackets, with indication of Species, Families and Orders.

Characiformes		
Crenuchidae		
Characidiinae		
		<i>Klausewitzia sp.</i> (66)
		<i>Elachocharax pulcher</i> (2340)
		<i>Microcharacidium eleotrioides</i> (30)
		<i>Ammocryptocharax minutus</i> (2)
		<i>Characidium sp.</i> (1)
		<i>Microcharacidium weitzmani</i> (481)
Curimatidae		
		<i>Curimatopsis evelynae</i> (34)
Lebiasinidae		

			<i>Pyrrulina latae</i> (1) <i>Nanostomus eques</i> (6) <i>Copella sp.</i> (27)
			Erytrinae
			<i>Hoplias malabaricus</i> (4)
			Characinae
			<i>Gnatocharax steindachneri</i> (1) <i>Hemigrammus stictus</i> (3) <i>Hemigrammus belloti</i> (31) <i>Hemigrammus</i> <i>worderwinkler</i> (5)
			Perciformes
			Ciclidae
			<i>Apistogramma sp.</i> (139) <i>Aequides pallidus</i> (13) <i>Crenicichla notoptalmus</i> (30)
			Eleotridae
			<i>Microphilipnus sp1</i> (102) <i>Microphilipnus sp2</i> (41)
			Gymnotiformes
			Hypopomidae
			<i>Microsternarchus</i> <i>billineatus</i> (74) <i>Hipopygus sp.</i> (147)
			Gymnotidae
			<i>Gymnotus anguilaris</i> (82)
			Siluriformes
			Pimelodidae
			<i>Nemuroglanis sp1</i> (117) <i>Nemuroglanis sp2.</i> (147)
			Scoloplacidae
			<i>Scoloplax dolicholophia</i> (76)
			Trichomycteridae

				<i>Trichomycterus sp1</i> (1)
				<i>Trichomycterus sp2.</i> (1)
				<i>Phreathobius sp.</i> (1)
				<i>Phisopixis aff. lira</i> (1)
			Loricariidae	
				<i>Acestridium discus</i> (3)
			Cyprinodontiformes	
			Rivulidae	
				<i>Rivulus aff. ornarus</i> (1)
			Synbranchiformes	
			Synbranchidae	
				<i>Synbranchus sp.</i> (7)

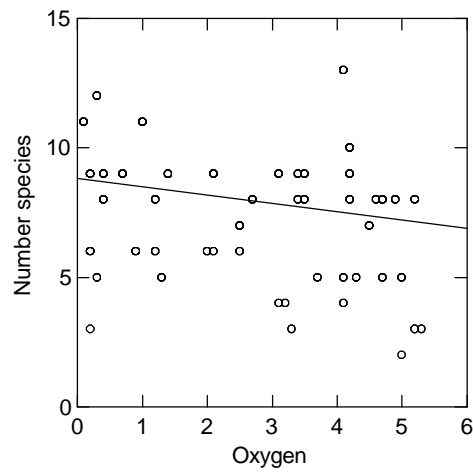


Figure 1. Relationship between number of fish species and dissolved oxygen in litter banks of Taramã-Mirim.  $r^2=232$ ,  $p= 0.000$ .

## Discussion

In small black water streams, the organic matter accumulated in marginal meanders is the main nutrient source. A diversity of organisms, around 122 species, including insects, larvae, crustaceans and fishes, can be found associated to these habitats (Walker, 1994). The environmental factors we analyzed (oxygen concentration, bank depth, bank volume, and flow speed) did not explain much the pattern of species distribution and abundance in the litter banks. Several studies have shown a relationship between the distribution of aquatic organisms and physical and chemical parameters like temperature, oxygen concentration, depth, flow speed, water chemistry and light. When anoxic conditions are created in *várzea* lakes, several fish species move to more favorable environments, and only resistant species stay in these conditions (Junk *et al.*, 1983). In macrophytes banks there is a high diurnal oxygen production and a high nocturnal consumption. Species associated to these banks move to open areas at night and use superficial aerial respiration (Saint-Paul & Soares, 1987).

Decomposition processes in litter banks are accelerated by the action of microorganisms and fungi, which together with crustaceans, fishes, insects and larvae reduce the amount of available oxygen. It is known that adaptive responses (morphological, physiological and genetic) are common in fish living in extreme conditions where environmental factors are much variable (Val, 1993). Laboratory experiments of oxygen consumption by the most abundant species revealed that they are very resistant to hypoxic conditions, what suggests that oxygen is not a limiting factor for the distribution of fishes in litter banks. The greatest number of species was found in places with the lowest oxygen concentration. Low oxygen conditions were found in the larger banks, which have a greater availability of resources, what might explain the use of the banks by so many species.

Another hypothesis is that these species stay in litter banks during the day and reduce their metabolism to avoid predators that do not exploit this habitat. This pattern was observed in aquariums mounted to simulate natural conditions. During the night several species were seen leaving the bank to explore neighboring habitats such as sand substrate.

### **Acknowledgements**

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**THE EFFECTS OF HYPOXIA AND HYDROGEN SULFIDE ON THE  
METABOLISM AND HEMATOLOGICAL PARAMETERS OF  
TAMBAQUI (*COLOSSOMA MACROPOMUM*)**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

In this study specimens of *Colossoma macropomum* were exposed to 12, 24, 48 and 96 h of sulfide and hypoxia after which samples of blood, heart, liver, and white muscle were taken for hematological, blood pH, and metabolites analysis. The acute exposures to hypoxia and sulfide were associated with elevated blood oxygen carrying capacity. The metahemoglobin concentration presented similar variation for both factors. The anaerobic metabolism of this species is an important adaptative mechanism to sulfide exposures in the natural environment. However, this mechanism is not specific to the toxic effects of this compound, since hypoxia in the tissues can be also induced by O<sub>2</sub> depletions in the water.

### *Experimental procedures*

Three groups of 24 fish (control, sulfide and hypoxia) were used to in experiments. To test the effect of sulfide, fish were exposed from 10 to 13  $\mu\text{M}$ . The sulfide concentration was determined by the methylene blue method while the water was maintained in normoxic levels. Exposure to hypoxia was carried out in a flow-through system where the oxygen levels were maintained by bubbling  $\text{N}_2$  (5mmHg). The control animals were maintained at normoxia (water  $\text{PO}_2 = 140$  mmHg) and at the acclimation temperature.

### *Sampling and analysis*

At 12, 24, 48 and 96 h of exposure, six fish of each treatment and control were removed. Blood samples were withdrawn for measurements of blood pH (pHe), hematological analysis (hematocrit, red blood cell count, hemoglobin concentration and metahemoglobin), glucose and lactate. After this procedure, fish were sacrificed and white muscle, liver and heart were used for analysis of glucose and lactate. The samples were deproteinized by 8% (w/v) PCA and the lactate and glucose were enzymatically determined. Glycogen was determined using 200 mg of white muscle or liver digested with KOH 6N following precipitation with ethanol and the glucosil units were analyzed by the enzymatic glucose oxydase.

Mean values of sulfide and hypoxic water groups in different times and within the groups were statistically compared to their controls using the Mann-Whitney U test. Differences were considered significant at  $p < 0.05$ .

### **Results and Discussion**

Sulfide and hypoxia induced an immediate increase of blood oxygen carrying capacity in tambaqui during the 48 h of fish exposure. After 96 h of exposure to both sulfide and hypoxia, the hematological parameters returned to values similar to those of the control, but a hemolytic anemia was observed in the fish exposed to sulfide. Probably, this anemia is not a specific effect of  $\text{H}_2\text{S}$ , but a general effect due to the confinement time of 96 h. Although the increase of metahemoglobin in the present study suggests a blood sulfide oxidation, it cannot be considered a sulfide mechanism detoxification, since the results are

similar to the fish exposed to hypoxia. The anaerobic metabolism is a possible physiological strategy for this species exposed to both hypoxia and high sulfide concentrations. The results obtained for liver and white muscle glycogen demonstrated that carbohydrate is an important energy source in this species during sulfide exposure. However, during exposure to hypoxia the liver and white muscle accumulated glycogen, and its importance to the organism should be investigated. Fish submitted to high sulfide concentrations presented an increase in blood, muscle and liver glucose levels until 24 h of exposure, probably as a consequence of anaerobic metabolism. Hypoxic fish only presented higher glucose levels in the blood. Hyperglycemia could indicate that the glycolysis is an important pathway for fish under such a condition. The blood pH values obtained for tambaqui after 24 h of exposure to hypoxia and sulfide indicate that the blood pH increases again near to the control values, probably to prevent the metabolic acidity. Although some differences were observed between the effects of hypoxia and sulfide, in general, the results presented similarity. It can be suggested that tambaqui is high sulfide tolerant as a consequence of its high tolerance to hypoxia.

#### **Acknowledgements**

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**ACIDOPHILIC FISH OF THE AMAZON RIO NEGRO:  
DO CALCIUM AND DOC AFFECT THEIR ACID TOLERANCE?**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

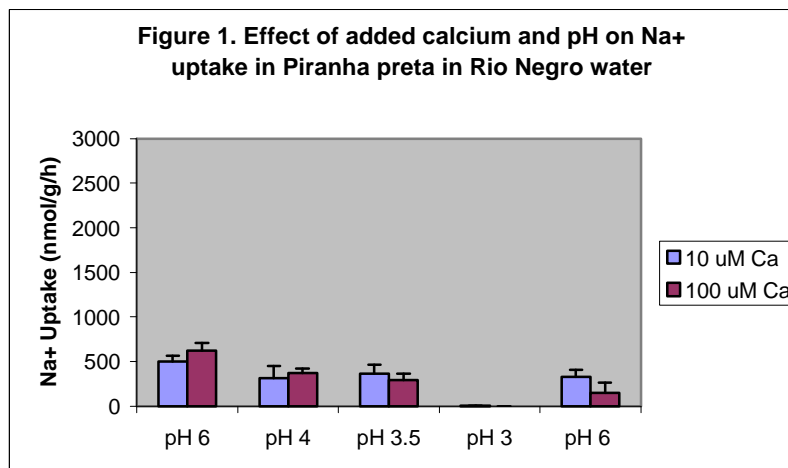
**Introduction**

Fish native to blackwater rivers such as the Rio Negro, Amazon, live in one of the most naturally acidic and dilute aquatic environments on earth. For example,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ion concentrations are frequently  $< 10 \mu\text{M}$  due to the immense precipitation and ion-poor soils. In addition, pH's in the range 3.5-4.5 are common in the forest streams due to the poor buffering capacity and the vast input of organic acids from decaying plant material. The extreme pH alone would be extremely toxic to the majority of freshwater fish. This is due to ionoregulatory failure at the gills as a result of a) inhibition of active ion uptake, and b) acceleration of paracellular ion losses. The latter is also normally exacerbated in very soft water (low  $\text{Ca}^{2+}$ ) due to the importance of external calcium in regulating the tight-junctional (i.e. paracellular) permeability of fish gills. It is possible that the ability of Rio Negro fishes to withstand these extreme conditions may be related to the presence of high levels of dissolved organic carbon (DOC). In addition, it is uncertain whether these fish have ionic effluxes that are dependent upon external calcium (Gonzalez et al., 1999). To examine these features of ion regulation we measured unidirectional ion fluxes ( $\text{Na}^+$  and  $\text{Cl}^-$ ) in adult Black piranha (*Pirhana preta*) resident to the blackwaters of the Rio Negro.

## Materials and Methods

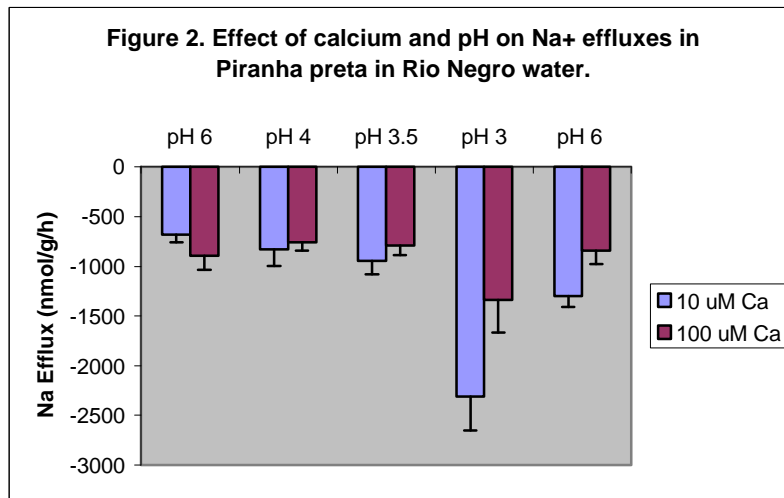
Experiments were conducted aboard the boat Amanai II during an expedition on the Rio Negro in December 1999. Animals were obtained from local fisherman and held in flowing Rio Negro water for several days prior to experiments. Fluxes were carried out with individual fish held in plastic containers holding 6 litres of aerated water. Fish were exposed to low pH (6.0-3.0) either in Rio Negro water with naturally high levels of dissolved organic carbon (DOC;  $>10 \text{ mg l}^{-1}$ ), or in well water containing negligible DOC but otherwise very similar ion contents. Unidirectional sodium influxes were measured by addition of  $^{22}\text{Na}$  radioisotope to the external medium and monitoring its disappearance over time (usually 1 hour flux periods). Net sodium fluxes were calculated from changes in total  $[\text{Na}^+]$  measured by flame photometry, and unidirectional effluxes were calculated as the difference between net flux and influx (Gonzalez et al., 1997).

## Results & Discussion



In Rio Negro black water, unidirectional and net  $\text{Na}^+$  fluxes were unaffected at  $\text{pH}'s \geq 3.5$ , and calcium (10 or 100  $\mu\text{M}$ ) had no additional influence. However, at pH 3,  $\text{Na}^+$  influx was abolished,  $\text{Na}^+$  efflux was doubled, and 100  $\mu\text{M}$  calcium

significantly reduced the impact on efflux by about 40% (Fig.1 & 2). Thus the protective influence of calcium appears to be pH-



dependent in these Rio Negro fish. Further experiments showed that Na<sup>+</sup> efflux was insensitive to DOC at pH 6 and 3.5 (when fluxes were measured in DOC-free well-water). However, Na<sup>+</sup> influx was inhibited by ~30% upon transfer from Rio Negro blackwater (DOC > 10 mg l<sup>-1</sup>) to water with low DOC (but with similar ionic content). This effect of DOC on the active Na<sup>+</sup> uptake mechanism suggests that the source of acidity (i.e. organic acids) may have some influence upon the acid tolerance of freshwater fish. The role of DOC in gill ion transport mechanisms clearly warrants further exploration if we are to understand the mechanisms behind the extremely high tolerance to acidity in these Amazonian fishes.

### Acknowledgements

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