

*Ion and
Acid-Base Regulation
in Fish*

SYMPOSIUM PROCEEDINGS

Steve McCormick

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International Congress on the Biology of Fish
Tropical Hotel Resort, Manaus Brazil, August 1-5, 2004

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International Standard Book Number (ISBN) 1-894337-48-4

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PREFACE

All teleost fish regulate ions in order to maintain plasma and intracellular ions at relatively constant levels. Anadromous and euryhaline species must have the capacity to reverse these mechanisms of ion transport. Similarly, all fish must regulate their acid-base balance, both in response to the external environment and internal generation of metabolic byproducts. Ion and acid-base regulation is critical to the normal function of most metabolic and physiological processes. The mechanisms for ion and acid-base balance are interrelated, and though we have a basic understanding of some of the mechanisms involved, it is clear that there is significant diversity among fish in the transporters involved, their localization and control. The papers in this symposium represent recent and important advances in our understanding of ion and acid-base physiology of fishes. The following areas of investigation are highlights of these contributions:

- Mechanisms of ion and acid-base regulation
- Characteristics and regulation of ion transporters
- Molecular approaches to ion and acid-base regulation
- Endocrine control of ion and acid-base regulation
- Toxicology and endocrine disruption of ion and acid-base regulation

We thank all of the contributors, both oral and posters, for contributing to the success of this symposium.

Symposium Organizers:

Steve McCormick, Conte Anadromous Fish Research Center, Massachusetts
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CONGRESS ACKNOWLEDGEMENTS

This volume is part of the Proceedings of the 6th International Congress on the Biology of Fish, held in Manaus, Brazil in August, 2004. Ten years have passed since the first meeting in this series was held in Vancouver, BC, Canada. Subsequent meetings were in San Francisco, California; Baltimore, Maryland; Aberdeen, Scotland; and again in Vancouver, Canada. From those meetings, colleagues from over 30 countries have contributed more than 2,500 papers to the Proceedings of over 80 Congress Symposia, all available for free viewing on the internet.

We would like to extend our sincere thanks to the many people who helped us organize the facilities and program for this 6th Congress.

The local arrangements team worked very hard to make this Congress a success. The leaders of those efforts were Vera Almeida Val, Adriana Chippari-Gomes, Nivia Pires Lopes and Maria de Nazare Paula Silva (Local Arrangements); Marcelo Perlingeiro (Executive Secretary) and Maria Angelica Laredo (Fund Raising). The enormous contribution of time and effort that was required has led to an unforgettable experience for the participants, thanks to the imagination, determination and dedication of this team.

Many sponsors helped ensure the success of the meeting through both monetary and in-kind contributions, including: Fundação Djalma Batista, Honda, Merse, Cometais, Turkys Aquarium, Banco da Amazônia, Banco do Brasil, FUCAPI, SEBRAE/AM, IDAM/SEPROR, FAPEAM, SECT-AM, SUFRAMA, PETROBRÁS, CAPES, FINEP, CNPq, the Physiology Section of the American Fisheries Society, UFAM - Federal University of Amazonas, Fisheries and Oceans Canada and INPA - National Institute for Research in the Amazon.

Travel arrangements were ably handled by Atlantic Corporate Travel (special thanks to Maria Espinosa) and Orcal Planet, and the venue for the meeting was the spectacular Tropical Hotel Conference Center in Manaus.

The Student Travel Award Committee of the Physiology Section of the American Fisheries Society, led by Michael Redding, evaluated 65 applications from 15 countries and awarded 40 Travel Grants, after an ambitious and trying fund-raising effort. Special thanks must go to the US Department of Agriculture, the US Geological Survey, US National Science Foundation and the World

Fisheries Congress for providing funds. In addition, the American Fisheries Society contributed books to be used as prizes for the best student papers.

The editorial team compiled the short abstracts into an abstract book and formatted and compiled the papers for the Symposium Proceedings. Thanks to Karin Howard, Christie MacKinlay, Anne Martin, Callan MacKinlay and Marcelo Perlingeiro.

In particular, we would like to extend a sincere 'thank you' to the organizers of the individual scientific Symposia and their many contributors who took the time to prepare a written submission for these proceedings. Their efforts are very much appreciated. We hope that their participation will result in new insights, new collaborations and new lines of research, leading to new papers to be presented at the 2006 Congress in St. John's, Newfoundland.

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**GENE REGULATION AND ION FLUX IN THE EURYHALINE
KILLIFISH *FUNDULUS HETEROCLITUS*
AFTER FRESHWATER TRANSFER**

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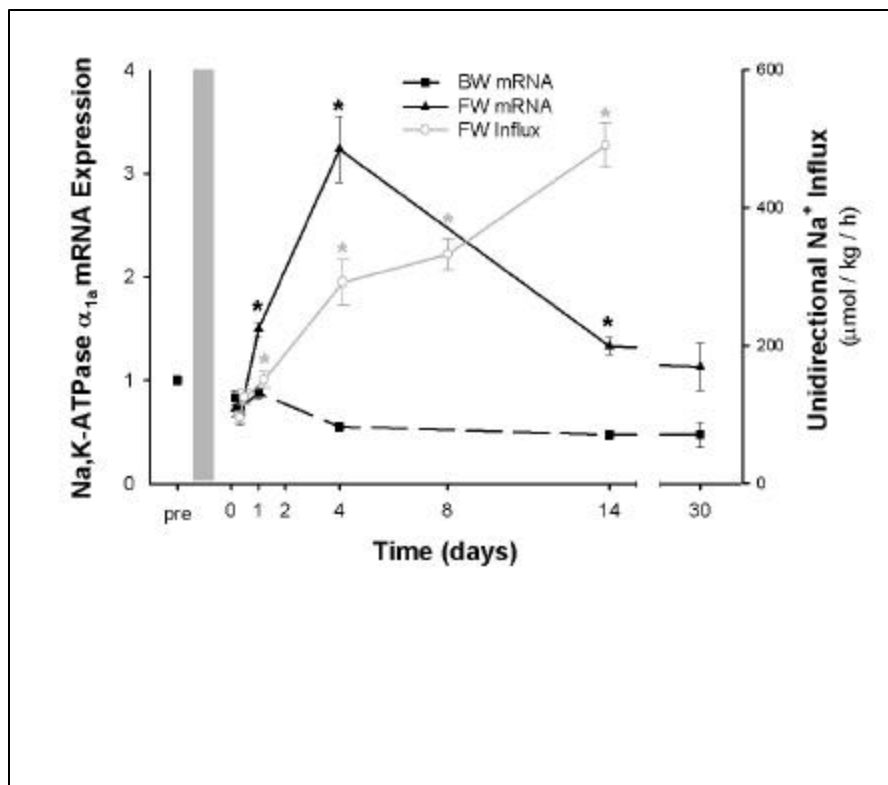
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EXTENDED ABSTRACT ONLY - DO NOT CITE

The euryhaline killifish *Fundulus heteroclitus* lives in brackish water estuaries and salt marshes along the east coast of North America, and thus experiences daily and seasonal variations in environmental salinity. As a result, killifish must frequently modulate the flux of Na⁺ and Cl⁻ to maintain ion balance. Recent work in our laboratories have examined the control of ion flux after transfer to dilute freshwater in this species. After transfer from near-isosmotic brackish water (10 ppt) unidirectional Na⁺ influx rate increases progressively, reaching levels 5-fold higher than immediately after transfer (Fig. 1) (Scott et al., 2004b). This is preceded by elevated Na⁺,K⁺-ATPase α_{1a} mRNA expression, suggesting that the resultant increase in enzyme abundance contributes to the observed changes in flux (Scott et al., 2004a). The direct role of Na⁺,K⁺-ATPase in mediating these changes in ion flux is further supported by data collected from different killifish populations. Intraspecific variation exists between northern and southern populations of killifish after freshwater transfer, such that northern killifish increase Na⁺,K⁺-ATPase expression and activity to a greater extent shortly after transfer. These differences in Na⁺,K⁺-ATPase regulation were shortly followed by reduced Na⁺ influx in southern killifish (Scott et al., 2004b).

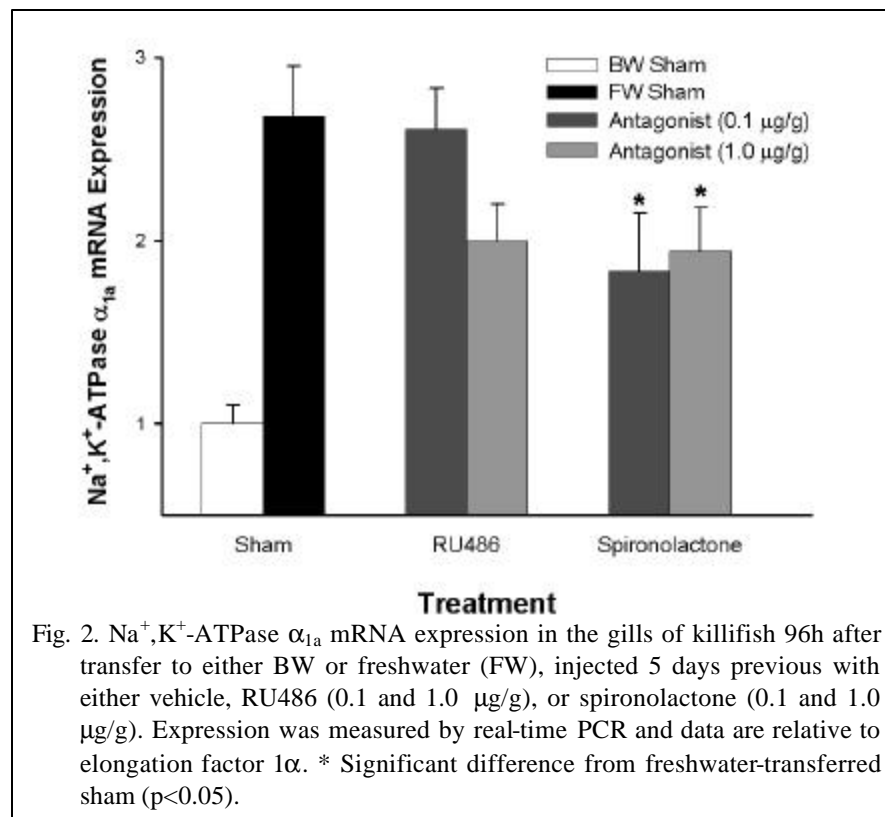
Due to the important role Na^+, K^+ -ATPase gene regulation appears to play in modulating Na^+ flux in freshwater, we examined how cortisol mediates the expression of this enzyme in killifish gills. In fish that were not subjected to salinity transfer and held in brackish water, increasing cortisol levels coincided with increased Na^+, K^+ -ATPase α_{1a} mRNA expression. In both sham and cortisol-injected ($75 \mu\text{g/g}$ fish weight, intraperitoneal injection) fish, expression of this gene increased 7-14-fold above untreated controls 24h and 96h after injection. Furthermore, increased expression persisted to 96h in sham-injected fish, even though plasma cortisol had returned to levels typical of untreated fish.

In fish that were transferred from brackish water to freshwater, cortisol antagonism inhibited the normal increase in Na^+, K^+ -ATPase α_{1a} mRNA expression (Fig. 2). Sham-injected fish transferred to freshwater increased expression of this gene nearly 3-fold above sham-injected brackish water controls. While RU486 (glucocorticoid [GR] receptor antagonist) injection had



no significant effect on expression, spironolactone (mineralocorticoid receptor [MCR] antagonist) inhibited Na^+, K^+ -ATPase α_{1a} mRNA expression by approximately 30% compared to freshwater-transferred sham-injected controls. These data suggest that cortisol may increase Na^+, K^+ -ATPase expression at least partially *via* MCR-mediated gene transcription.

We localized MCR in killifish gills by immunocytochemistry using a polyclonal antibody raised against human MCR. The receptor localized primarily to Na^+, K^+ -ATPase-immunoreactive cells in both apical and sub-apical regions of the gills. Furthermore, staining of the gills with this antibody was distinct from staining patterns observed for GR. It is therefore unlikely that the MCR antibody was cross-reacting with killifish GR. Distinct localization of this receptor to Na^+, K^+ -ATPase-immunoreactive cells supports current suggestions that MCR mediates the stimulatory effect of cortisol on ion absorption across fish gills in



freshwater (Sloman et al., 2001).

In summary, our work suggests an important role for Na⁺,K⁺-ATPase gene expression in mediating Na⁺ uptake across fish gills. Cortisol appears to regulate whole-gill Na⁺,K⁺-ATPase α_{1a} mRNA abundance, and likely does so in part *via* MCR. Future work will assess whether the effect of cortisol in fish gills is due to increased transcription of Na⁺,K⁺-ATPase α_{1a} mRNA or proliferation of Na⁺,K⁺-ATPase α_{1a} expressing cells.

Acknowledgements

The authors would like to thank Drs. Toyoji Kaneko and Bernadette Ducouret for kindly supplying Na⁺,K⁺-ATPase and GR antibodies, respectively. We would also like to acknowledge Dr. Jeff Richards and Linda Diao for technical help and support, and funding from NSERC. Some of the work presented here has previously appeared in manuscript form (see references).

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**RAPID ADAPTATION OF THE KILLIFISH (*Fundulus*
heteroclitus)
TO SALINITY CHANGE**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The euryhaline killifish *Fundulus heteroclitus* lives in estuaries and experiences rapid salinity fluctuations. Killifish (northern population) acclimated to 10% seawater (NaCl ~ 55 mM) and transferred to either moderately hard freshwater (FW; NaCl ~ 0.9 mM) or 100% seawater (SW) achieved rates of Na⁺ and Cl⁻ exchange, measured radioisotopically, which were approximately stable from 12 h through 7 days post-transfer (Wood and Laurent, 2003). Hourly flux rate measurements were examined for the 0-12 h post-transfer period in detail. Immediately after transfer to FW, Na⁺ influx rate falls, while Cl⁻ influx rate drops to zero. Thereafter, Na⁺ influx rate increases moderately and achieves

stable levels typical of FW-acclimated fish by 2 h, while Na^+ (Fig. 1) and Cl^- efflux rates decrease progressively, becoming stable by 8 h. However, Cl^- uptake from FW does not occur and remains negligible, even after long-term FW acclimation. Thus unlike Na^+ , whole body Cl^- balance remains negative, suggesting that Cl^- homeostasis during freshwater residency must be maintained by dietary rather than gill uptake, similar to the well-known situation of the eel in freshwater (e.g., Grosell et al., 2000). After transfer to 100% SW, Na^+ (Fig. 1) and Cl^- efflux rates rise progressively, achieving stable levels typical of SW-acclimated fish by 8 h. Na^+ and Cl^- influx rates are initially greatly elevated, but

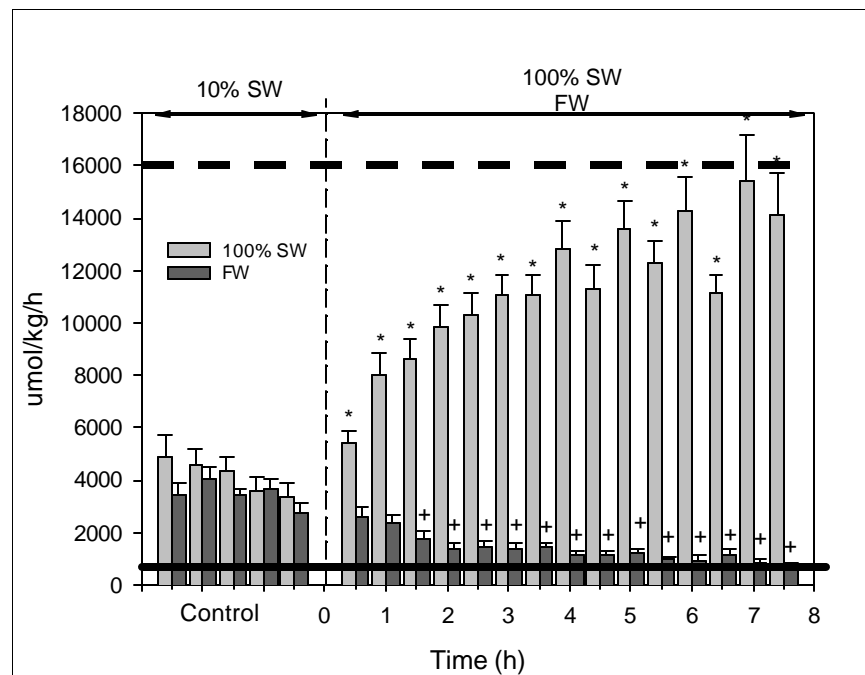


Figure 1. Unidirectional Na^+ efflux rates after transfer of killifish from 10% SW to either FW or 100% SW. The horizontal lines indicate the rates in fish long-term acclimated to the two media.

then decrease to stable but still elevated levels typical of SW-acclimated fish by 3 h. Thus overall, acclimated rates of influx and efflux are achieved rapidly, at

most within 8 h post-transfer.

Gill surface morphology changes with similar rapidity after transfer to FW, exposing chloride cells whose apical surfaces are equipped with microvilli. In contrast, gill morphology remains unaltered after transfer to SW. Gill chloride cell mitotic rate, measured with the BRDU technique, increases greatly 10-14 h after transfer to FW, but remains unchanged after transfer to SW.

In view of the more marked physiological and morphological changes upon transfer to FW than to SW, we focussed on gene expression (by real-time PCR analysis) and biochemical changes accompanying the FW transition. Of particular interest was a comparison of gill versus opercular tissue, for the latter has often been used as a surrogate model for the gill, yet it fails to actively take up Na^+ from FW when mounted *in vitro* (Marshall et al., 1997). In both tissues, there is rapid, persistent (12 h – 7 d) up-regulation of 14-3-3.a mRNA and down-regulation of CFTR mRNA, which would comprise a mechanism to reduce Cl^- efflux. Furthermore in both tissues, Na^+, K^+ ATPase activity remains unchanged after transfer to FW, in accord with unchanged Na^+, K^+ ATPase $\forall 1\alpha$ mRNA levels. \forall type H^+ ATPase and NBC (NaHCO_3 co-transporter) mRNA levels remain similarly unchanged as a result of transfer, but both of the latter are expressed at much lower absolute levels in the opercular epithelium than in the gill tissue. NHE-2 (apical Na^+/H^+ exchanger-2) mRNA is transiently up-regulated in the gills at 12 h after transfer to FW, yet is undetectable in the opercular epithelium. There is also a rapid, persistent up-regulation of CA-2 (carbonic anhydrase-2) in gills after transfer, but a down-regulation of CA-2 in the opercular epithelium (Fig. 2), as well as a much lower absolute expression level of this gene in the latter.

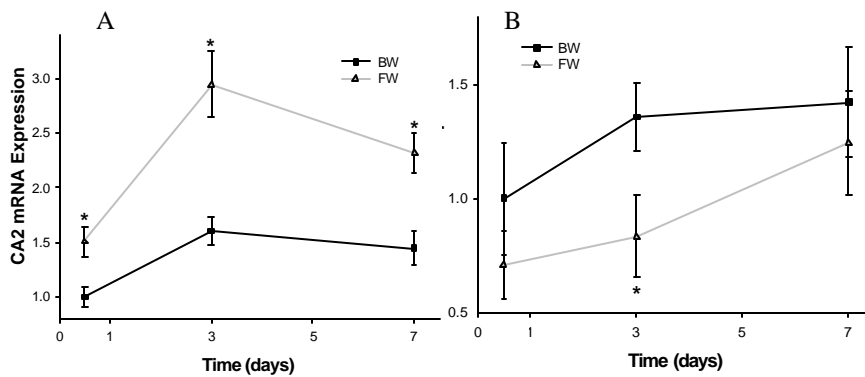


Figure 2. Changes in CA-2 (carbonic anhydrase-2 mRNA levels) in (a) gills and (b) opercular epithelium of killifish after transfer from 10% SW to FW. BW data represent control animals left in 10% SW.

Taken together, these observations likely explain the mechanism of active Na^+ uptake at the gills and its absence at the opercular epithelium. Furthermore, NHE-3, often implicated in acid-base regulation, is down-regulated in the gills yet up-regulated in the opercular epithelium after transfer, reinforcing the view that these two tissues behave very differently in FW.

Acknowledgments

Supported by NSERC Discovery Grants to CMW and PMS and NSF IBN-0111073 to JBC. We thank Linda Diao for technical support, as well as Toyoji Kaneko and Shigehisa Hirose for kindly supplying antibodies.

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IONOREGULATION
IN THE GLASS EEL (*ANGUILLA ANGUILLA*)

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Introduction

The European eel (*Anguilla anguilla*) has a facultative catadromous life history. The juvenile glass-eel stage makes the migration into freshwater and the important transition from hypo-osmoregulation to hyper-osmoregulation. Despite the economic importance of the glass eel fishery to aquaculture and the significance of this freshwater transition to river system recruitment, relatively little attention has been directed at the osmoregulatory physiology of this life history stage (see Wilson et al. 2004).

Two key ATPase associated with iono regulation in sea water and freshwater are Na⁺,K⁺-ATPase and V-ATPase respectively. Na⁺,K⁺-ATPase is the driving force for the elimination of Cl⁻ in conjunction with the NKCC and a Cl⁻ channel protein in seawater fishes and is also involved in Na⁺ uptake in freshwater fishes although levels of activity tend to be lower in freshwater fishes. The V-ATPase is believed to drive Na⁺ uptake by creating a favourable electrochemical gradient. In order to determine if there are seasonal changes in the expression of these two ATPase at the point of estuarine entry, monthly sampling was conducted throughout the year.

The regulation of Na⁺,K⁺-ATPase activity has been shown to be under endocrine control (McCormick 2001). The steroid hormone cortisol has been shown to

increase levels of Na^+, K^+ -ATPase in the gill as well as the number of branchial mitochondria-rich cells (MRCs) and has been called the “seawater hormone” although it does have similar effects in freshwater salmonids. In the present study, the role of cortisol in FW adaptation was investigated by looking at the effects of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) blockade using the antagonists mifepristone (RU-486) and spironolactone, respectively.

Material and Methods

Glass eels (*Anguilla anguilla* L.) were collected from the mouth of the River Minho monthly during the new moon phase over a 2 year period and transported to the laboratory where they were kept in small temperature controlled holding tanks. Some animals were immediately sampled as part of the monthly sampling regime. When numbers were sufficient, the remainders were kept in the lab for further experiments. No attempt was made to feed animals during holding. Animals were anaesthetized and given intra-peritoneal injections of either coconut oil (CO; $0.5 \mu\text{l} \cdot 0.1 \text{g bw}$); CO+ $50 \mu\text{g} \cdot \text{g}^{-1}$ cortisol (F); CO + $100 \mu\text{g} \cdot \text{g}^{-1}$ RU-486 (mifepristone); or + $100 \mu\text{g} \cdot \text{g}^{-1}$ spironolactone (Sigma Chemical Co.). After 10 days animals were sampled. (Wilson et al. 2004).

Results and Discussion

There were no seasonal differences in the activity of branchial Na^+/K^+ -ATPase or V-ATPase. During the summer months, however, late stage glass eels (more heavily pigmented and have spent a longer time in the estuary) Na^+/K^+ -ATPase activity was significantly lower than in early stage glass eel (new arrivals to the estuary) from the same sampling. V-ATPase activities were not different. In additional experiments comparing FW and SW acclimated glasseels, V-ATPase activities were also not different. These results are altogether not too surprising since Na^+ uptake rates in eels have been found to be quite low compared to trout that show marked changes in V-ATPase expression.

In the implant studies, a drop in branchial Na^+, K^+ -ATPase activity was associated with acclimation to freshwater in control glass eels given CO implants (Fig.1; see also Wilson et al. 2004). This drop was also evident in glass eels give cortisol or RU-486 implants although in the latter group the drop was not significant ($P=0.069$). In glass eels given spironolactone implants there was

clearly no change ($P=0.988$). These data suggest that cortisol might have a role in the down regulation of Na^+/K^+ -ATPase activity through the mineralocorticoid receptor.

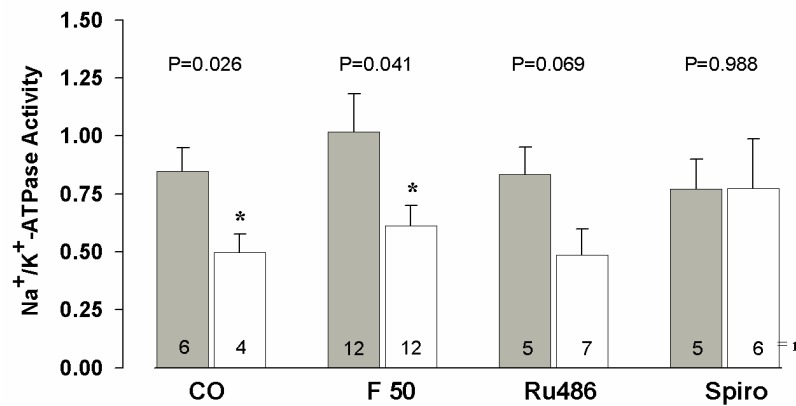


Figure 1. Ouabain sensitive Na^+/K^+ -ATPase activity measured in crude gill homogenates of glass eels given ip implants of coconut oil (CO), cortisol, RU486 or spironolactone after 7d in SW or FW.

Acknowledgements

This work is supported by an FCT PRAXIS XXI grant (POCTI/BSE/34164) and BIC support to PNRs, AVF and PDB. We would like to thank Drs. M.A. Reis Henriques and L. Guilhermino for use of equipment and our fisherman Eduardo Martins.

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Fish. Aquat. Sci.***61**:432-442

**SLC26A ANION EXCHANGER EXPRESSED
IN RAINBOW TROUT (*Oncorhynchus mykiss*) KIDNEY**

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Introduction

The teleost kidney plays a major role in the excretion of excess divalent ions in seawater and reabsorption of monovalent ions and acid-base regulation in fresh water, respectively. However, the molecular mechanisms of ion transport in the teleost kidney remain unclear. The SLC26A is a family of recently discovered highly versatile anion exchangers. The ten-member SLC26 gene family encodes anion exchangers capable of transporting a wide variety of monovalent and divalent anions. Some of the family members have been reported to be located in mammalian kidney, and specifically involved in sulfate, chloride, or bicarbonate transport (Mount, D. B. and Romero, M. F., 2003). There is high possibility that a SLC26A anion exchanger plays a major role in secretion of divalent ions in seawater-adapted fish. Although the SLC26A6 homolog has been cloned from Japanese eel, and evidence for ion transport in fish kidney has been discovered (Nishimura, H. et al., 1983; Renfro, J. L. and Pritchard, J. B., 1983), the functional, physiological and

morphological analysis of the fish kidney has never been studied in fish. In the present study, full-length SLC26A1 cDNA, which is a known sulfate transporter in mammalian kidney, was cloned. We found that it is primarily expressed in the trunk kidney of rainbow trout.

Materials and Methods

cDNA cloning of SLC26 A anion exchanger was performed from seawater-adapted rainbow trout trunk kidney using RT-PCR followed by 5' and 3' RACE methods. Expression of the SLC26A anion transporter was examined in different tissues from seawater-adapted rainbow trout using Northern blot analysis.

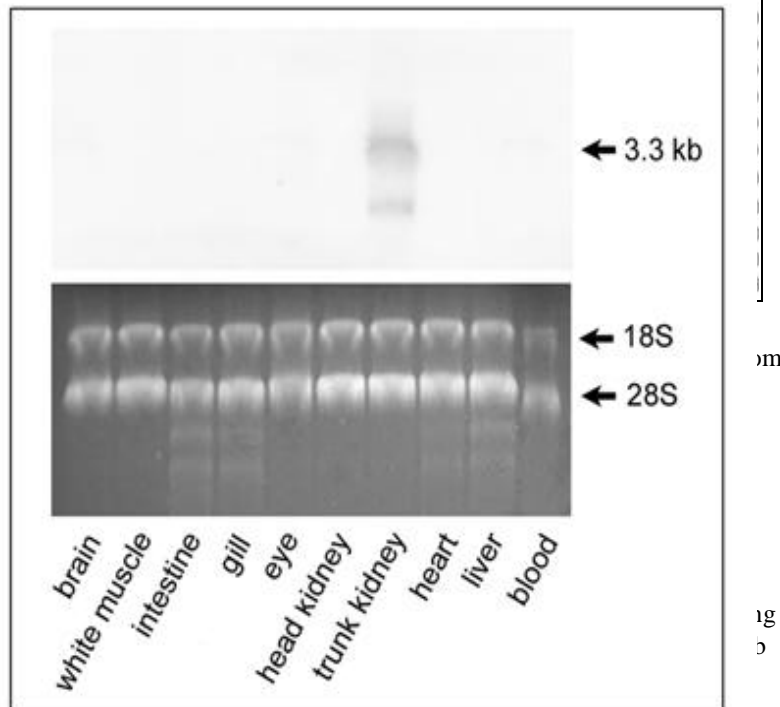


Figure 2. Tissue distribution of SLC26A1 mRNA determined by northern hybridization in seawater-adapted rainbow trout.

Expression was primarily detected in trunk kidney.

Conclusions

SLC26A1 anion exchanger, which in mammals exchanges sulfate and oxalate for chloride, was cloned from seawater-adapted rainbow trout trunk kidney. The cloned SLC26A1 anion exchanger expression was detected in only in the trunk kidney of seawater-adapted rainbow trout. These results suggest that SLC26A1 expressed in seawater-adapted rainbow trout trunk kidney may play a major role in the excretion of excess sulfate as divalent ions exist at toxic levels in seawater.

Acknowledgement

F. K. was supported by a Research Fellowship awarded by the Japan Society for the Promotion of Science for Young Scientists. This work is support by an NSERC discovery grant to G.G.G.

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**MECHANISMS OF SALINITY TOLERANCE IN CALIFORNIA
MOZAMBIQUE TILAPIA (*OREOCHROMIS MOSSAMBICUS* X *O.
UROLEPIS HORNORUM*) EXPOSED TO SALINITIES GREATER THAN
SEAWATER**

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Summary

The salinity tolerance of the California Mozambique tilapia, a current resident of the hypersaline Salton Sea in southeastern California, was investigated by gradually acclimating fish to progressively increasing salinities from 35 to 95 g/l (in 10 g/l increments for five days per increment), while physiological, biochemical, and morphological indicators of osmoregulatory stress were measured. Tilapia survived exposure to all salinities with only minor mortality in the 85 g/l treatment. Our results indicate that tilapia maintain internal osmotic balance without any change in drinking rate or branchial Na⁺, K⁺-ATPase activity when exposed to salinities up to 65 g/l for five days. At 65 g/l salinity or greater, there were changes in chloride cell turnover, drinking rate, and Na⁺, K⁺-ATPase activity; followed by increases in plasma osmolality and ion levels. We conclude that this tilapia hybrid maintains its internal environment by decreasing its epithelial permeability, perhaps as an acute measure for dealing with short term increases in environmental salinity, but when ambient salinity is too great, strategies of osmoregulation such as increased drinking rate and increased branchial Na⁺, K⁺-ATPase activity become apparent. Further

investigation may also determine if the duration of exposure plays a role in osmoregulatory strategy.

Introduction

The Salton Sea is a 980 km² hypersaline lake in the desert southeast of California. This inland sea formed in 1905-06, when water, accidentally diverted from the Colorado River, flooded the Imperial and Coachella Valleys. With no outflow and a high rate of evaporation, over the past 100+ years, the Salton Sea has become increasingly saline; today the salinity is near 43 g/l and increasing at a rate of about 0.3 g/l/year. For the past thirty years, the Salton Sea has boasted a substantial sport fishery, through transplants of fish from the Sea of Cortez, or in the case of the California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*), by escape from local fish farms; however, recent large-scale mortality events have been partially attributed to salinity. While other factors such as large scale fluctuations in temperature, periods of hypoxia/anoxia, metal toxicity, and high sulphide levels (Watts et al 2001) may all contribute to the declining fish population, salinity has been identified as the key environmental factor and attempts to assess the effects of its continual increase on the fish population are currently being explored. If salinity of the Salton Sea continues rising at its current rate, it will exceed the tolerance levels of its inhabitant species, which will lead to the complete collapse of the fishery.

The California Mozambique tilapia is a hybrid species between the Mozambique, which is highly euryhaline, and the Wami tilapia, about which very little is known (Costa-Pierce & Doyle 1997). Mozambique tilapia are found in freshwater and estuarine systems, but in experimental conditions, have tolerated salinities as high as 120 g/l (Stickney 1986). The objective of this study was to investigate the salinity tolerance of commercially available tilapia hybrid, in order to gain insight into its hypoosmoregulatory ability and establish a model of salinity tolerance for the Salton Sea species. Tilapia were gradually exposed to a progressive increase in salinities 35 to 95 g/l (in 10 g/l increments for 5 days per increment) and physiological (plasma osmolality, [Na⁺], and [Cl⁻], oxygen consumption, drinking rate, hematocrit, mean cell hemoglobin concentration, and muscle water content), biochemical (Na⁺, K⁺-ATPase activity), and morphological (number of mature, accessory, immature and apoptotic chloride cells) indicators of osmoregulatory stress were measured.

Materials and Methods

Juvenile tilapia hybrids (36.05 ± 0.41 g) were donated by Pacific Aquafarms in Niland, CA, acclimated to seawater over a four week period, and divided into seven tanks. Salinity was increased in each tank by 10 g/l every five days via a three-quarter water change. At each salinity (35-95 g/l) one tank was sampled, where seven fish were removed at 0, 3, 24, and 120 h; yielding experimental exposures to 35, 45, 55, 65, 75, 85, and 95 g/l at 25°C.

Each sampling time involved collection of blood for hematocrit (Hct), hemoglobin concentration (Hb), and subsequent mean cell hemoglobin concentration (MCHC), and for determination of plasma osmolality, $[Na^+]$, and $[Cl^-]$. Posterior epaxial muscle was removed and dried to determine the percent muscle water content, and gill and gut tissue was taken for Na^+ , K^+ -ATPase activity (Gibbs & Somero 1990). At 120 h, additional gill arches were taken and preserved in fixative for analysis of morphology with transmission and scanning electron microscopy. Oxygen consumption and drinking rates were determined in fish exposed to increased salinity for a two week period, at salinities of 35, 55, 75, or 95 g/l. Oxygen consumption was determined according to the methods of (Gonzalez & McDonald 1994), and drinking rate was measured using a modified method of (Wilson et al 1996).

Results

Oxygen consumption fell in a linear fashion with ambient salinity (Fig. 1), and was significantly decreased relative to seawater values at 75 g/l salinity. No significant changes in muscle water content (pooled mean \pm SE = $80.59 \pm 0.12\%$), Hct ($30.42 \pm 0.29\%$), Hb (1.04 ± 0.01 mM), or MCHC (3.44 ± 0.03 mM calculated as $[Hb]/(Hct/100)$) were observed.

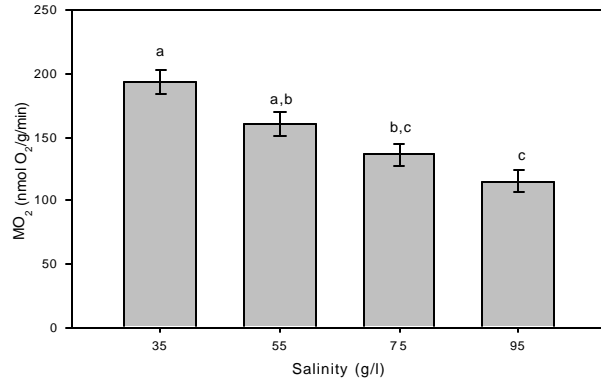


Figure 1: The effect of salinity on oxygen consumption rate. Different letter indicate statistical differences by one-way ANOVA, $p < 0.05$. (Taken from Sardella et al. 2004)

Plasma osmolality was significantly increased at 75 g/l salinity at 24 and 120 h relative to seawater values, and thereafter; plasma $[Na^+]$ and $[Cl^-]$ followed similar trends (Fig. 2a). No changes in Na^+ , K^+ -ATPase activity were seen at 45 or 55 g/l, but levels increased relative to seawater values at 24 h in fish exposed to 65 g/l, returning to control values by 120 h. Na^+ , K^+ -ATPase activity was elevated at all times sampled from 75-95 g/l fish (Fig. 2a). Drinking rate remained relatively unchanged between 35 and 55 g/l-exposed fish, but was significantly increased in 75 g/l-exposed fish (Fig. 2a). The morphology of the branchial epithelium remained largely unchanged between 35 and 55 g/l-exposed fish, however, at 55 g/l, the first observation of accessory cell-chloride cell interdigitated junctions was observed. There were dramatic changes in the epithelium at salinities following this change, including increases in chloride cell turnover rate, number of apical pits, and number of apoptotic chloride cells (Fig. 1b).

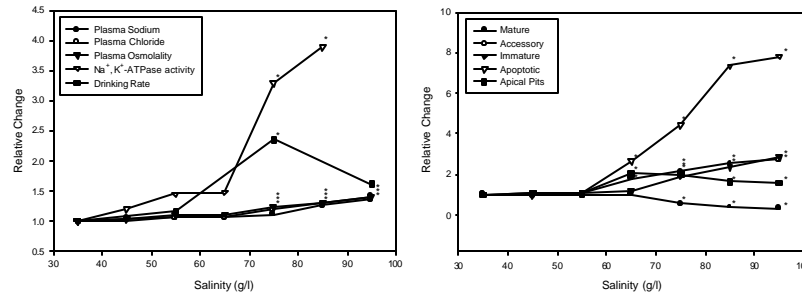


Figure 2: Indices of change relative to values measured in seawater-acclimated animals for a) Plasma osmolality, $[Na^+]$, and $[Cl^-]$, Na^+,K^+ -ATPase activity and drinking rate, and b) numbers of apical pits and chloride cell sub-types with increasing salinity (* absolute values statistically significant from seawater-acclimated fish, $p < 0.05$). (Modified from Sardella et al 2004b).

Discussion

Oxygen consumption fell in a linear fashion with increasing environmental salinity. Decreases of this nature are commonly observed in teleost species, and probably reflect a reduction in activity as well as down regulation of non-essential systems (Swanson 1998). The lack of change in the indicators of osmoregulatory stress may indicate that at five days of salinity exposure at constant temperature, salinities up to 55 g/l are tolerable without any change in strategy (Fig 1). Tilapia may survive acute exposures to increased salinity by reducing branchial permeability; however, if salinity stress is high due to increased salinity (55 g/l+ in this study), or extended duration of exposure (Cioni et al 1991), this mechanism may not be sufficient, and more conventional strategies of osmoregulation, such as increased drinking rate and Na^+, K^+ -ATPase activity, are then observed. The pattern of change in morphological parameters is consistent with the hypothesis that tilapia do not change osmoregulatory strategy after five days of exposure to salinities below 55 g/l. It further adds to the model; changes in morphology preceded changes in physiological or biochemical indicators of osmoregulatory stress, and the dramatic changes in those parameters occurred following an increase in branchial membrane permeability (as determined by depth and area of apical tight junctions) (Cioni et al 1991). Furthermore, it has been previously observed, using a direct transfer to hypersalinity protocol and electrophysiological parameters, that Mozambique tilapia dramatically reduce

the conductance of chloride cell-accessory cell junctions following transfer (Kultz & Onken 1993).

Based on our results, the most sensitive indicator of osmoregulatory stress in this species after five days of exposure to progressively increasing salinities is the number of apoptotic chloride cells in the branchial epithelium. The next step in constructing a model by which to compare wild fish caught from the Salton Sea is to assess the changes in these osmoregulatory patterns when the duration of exposure to hypersaline conditions is extended. Furthermore, the additive effects of temperature, dissolved oxygen level, toxic metals, and sulphide concentration also need to be considered; both increases and decreases in temperature have been shown to reduce the osmoregulatory capacity of this tilapia hybrid (Sardella et al 2004a). Once further data is gathered, the most sensitive indicators observed in our laboratory studies can be investigated in wild caught fish, and the osmoregulatory status of the Salton Sea population may be determined.

Acknowledgements

This work was supported by a grant from the Salton Sea Authority, the Salton Sea Science Office, and U.S. Bureau of Reclamation, and NSERC Discovery Grant to CJB. We wish to thank Doug Barnum and Ray Stendell (SSSC) for their input and general interest in the project, as well as Phil Davis and Erica Dale (SDSU) for their efforts and fish care, and Steve Barlow (SDSU) for help with microscopy. Finally, we greatly thank Colin Bornia and Bill Engler of Pacific Aquafarms for their kind donation of fish, along with their input and interest in this project.

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**NATRIURETIC PEPTIDE SIGNALLING IN THE GILLS
OF FRESHWATER AND SEAWATER FISHES**

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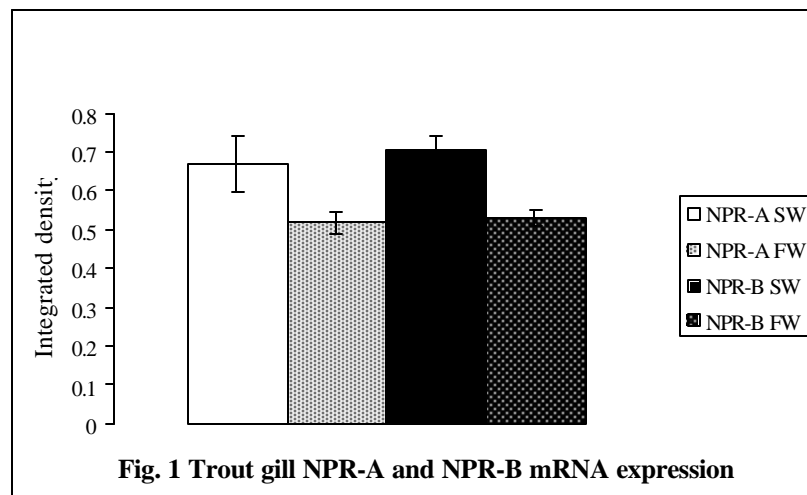
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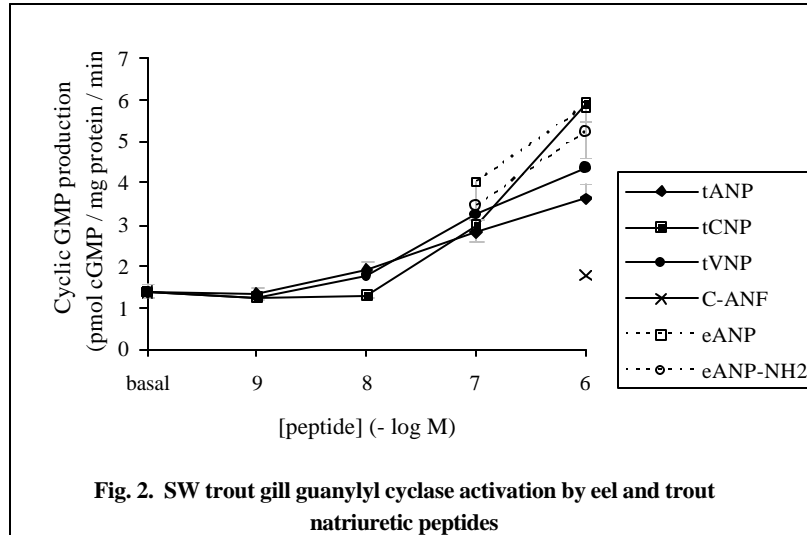
The natriuretic peptide system of fishes is implicated in the maintenance of osmotic and cardiovascular homeostasis through effects on tissues including the blood vessels, heart, gut, brain, kidneys and gills. While there are a number of extracardiac sites of natriuretic peptide expression, the major circulating forms are secreted from the heart. For some years, the known suite of natriuretic peptides in fishes had been limited to atrial natriuretic peptide (ANP), ventricular natriuretic peptide (VNP) and C-type natriuretic peptide (CNP). These peptides exert their effects via four natriuretic peptide receptors (NPR): the guanylyl cyclase (GC) receptors, NPR-A (for which 2 subtypes have been identified in medaka, *Oryzias latipes*) and NPR-B, and the non GC receptors, NPR-C and NPR-D (Toop and Donald, 2004). Recently, however, other members of the natriuretic peptide family have been identified in fishes, including four separate CNP genes (CNP-1, CNP-2, CNP-3 and CNP-4), which differentially activate the two NPR-A subtypes in medaka, and NPR-B (Inoue et al., 2003). Also, BNP has now been found in the sturgeon, *Acipenser transmontanus* (Kawakoshi et al., 2004) and is presumably present in other fish

species. However, not all fish appear to possess the full complement of natriuretic peptides. Consequently, the history of natriuretic peptides in bony fishes is more complex than originally thought.

We have studied the mRNA expression of NPR-A and NPR-B in the gills of freshwater (FW) and seawater-acclimated (SW) rainbow trout, *Oncorhynchus mykiss*, using standard semi-quantitative RT-PCR, with NPR expression normalised against the expression of β -actin. (Fig. 1). NPR-B expression was greater in SW than in FW gills (Student's t-test, $p < 0.05$). NPR-A expression between treatments did not differ statistically, although the trend is also for greater expression in SW. These data contrast with those from the Japanese eel, *Anguilla japonica*, in which NPR-B mRNA expression was greater in FW gills (Katafuchi et al., 1994).



We also examined the abilities of eel and trout ANP, amidated eel ANP, trout VNP and trout CNP (now known to be a CNP-1) to stimulate GC activity in the gills of the same fish using an in vitro GC assay, followed by measurement of cGMP (Fig. 2, Callahan et al., 2002).



All natriuretic peptides stimulated GC activity above basal at concentrations of 10^{-7} M and 10^{-6} M. In SW gills, trout CNP elevated GC activity to a greater extent than trout ANP or VNP, but was not different from the eel peptides. In FW gills (data not shown), trout CNP elevated GC activity to a greater extent than trout ANP. Salinity had no effect on natriuretic peptide stimulated GC activities. In medaka, CNP-1 stimulated GC activity in the NPR-A homologue, OIGC7, at 10^{-7} M and 10^{-6} M but not the second NPR-A homologue, OIGC2, which was activated only by CNP-3 (Inoue et al., 2003). It is possible that trout CNP is activating both NPR-A (an OIGC7 type) and NPR-B at the two higher concentrations, accounting for the increased cGMP production by CNP over trout ANP and VNP. We have not obtained sufficient trout NPR-A cDNA sequence to determine which NPR-A we have partially cloned. The enhanced GC receptor mRNA expression in SW gills over FW gills (Fig. 1) does not appear to correlate with results from the GC assays (Fig. 2 and Callahan et al., 2002). Probably, this is because the mRNA is not reflecting the protein receptor pool, or, the GC assays are not sensitive enough to reveal subtle changes in NPR complements in different salinities.

The promiscuity of trout NPRs has previously been noted (Toop and Donald, 2004). This is in contrast to the strict selectivity of Japanese eel NPRs for eel natriuretic peptides (Takei et al., 1989; Kashiwagi et al., 1999, cited in Callahan et al., 2002). We have found that this promiscuity of natriuretic peptides for homologous and heterologous peptides is common among fish species. We have also observed, along with others, that CNP is the strongest stimulator of GC activity in the gills (Toop and Donald, 2004). Now that we know bony fishes can possess up to four CNPs, plus ANP, VNP and BNP, it is perhaps easier to understand why gill NPR-GC can be stimulated almost equally by a range of natriuretic peptides. That CNP is the most potent stimulator of GC at higher concentrations is probably due to its ability to activate several receptor types. Given the complex nature of the natriuretic peptides in bony fish, it will be interesting to see if there are other NPR in the gills of fishes.

Acknowledgements

We thank Professor Yoshio Takei of the Ocean Research Institute, University of Tokyo for his kind gift of trout and eel peptides and Dr. Mark Powell, School of Aquaculture, University of Tasmania, for acclimating and providing the trout.

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**CHANGES IN EXPRESSION
OF HYPO-OSMOTIC HORMONE GENES
IN PRE-SPAWNING SALMON
DURING HOMING MIGRATION**

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The previous studies indicate that neurohypophysial hormones and prolactin are involved in hypo-osmotic regulation in salmonids. Hence, changes in the amounts of mRNAs for neurohypophysial hormones and pituitary hormones were examined in pre-spawning chum salmon (*Oncorhynchus keta*) during homing migration toward their natal hatchery, to understand molecular bases of freshwater adaptation in salmonids.

Since gonadal maturation accompanied a loss of salinity tolerance in many salmonids, that is, increased plasma Na⁺ levels and high mortality were observed when homing fish were retained in seawater (SW)(see Saito et al., 2001), we compared two salmon stocks that return short and long rivers to discriminate effects of hypo-osmotic stimulation from influences of sexual maturation. The former is the Otsuchi River, in which fish need only a day to reach their natal hatchery 4 km upstream to the mouth of river, and the latter is the Ishikari River, in which salmon require 2 to 3 weeks to arrive the natal hatchery.

Chum salmon were sampled at several areas along their homing pathway from the coastal sea to the spawning ground in august at least for three years to

eliminate year to year differences under influences of body size, nutritional condition, gonadal maturity and oceanographic conditions. In addition, we examined effects of hypo-osmotic stimulation by transfer from SW to fresh water (FW). Maturity of homing fish was estimated by gonadosomatic index, gonadal state, nuptial color and plasma steroid hormone levels. Salinity tolerance was estimated by plasma Na^+ and mortality in SW environment.

The absolute amounts of mRNAs for neurohypophysial hormones, vasotocin and isotocin, and pituitary hormones including gonadotropin subunits and growth hormone (GH)/prolactin (PRL)/somatolactin (SL) were determined by quantitative dot-blot analyses or real-time polymerase chain reaction (PCR) using cDNAs as the standards. Changes in gene expression were assessed by the variations of mRNA amounts.

Homing fish of Otsuchi stock were fully matured, and showed high plasma Na^+ levels and high mortality in the SW environment in the years when the warm current dominated in the coastal sea. Hypothalamic vasotocin mRNA levels increased in the males, but decreased in the females (Fig. 1). GH and PRL levels in the pituitary increased when fish entered the river (Fig. 2). The SW-FW transfer experiment in the same year showed that vasotocin mRNA levels markedly increased in the SW-retained control males, whereas the mRNA levels were decreased by SW-transfer in both sexes. Interestingly, the amount of PRL mRNA was elevated in the SW-retained fish. In the SW-FW transfer experiment performed in the year when the branch of cold current is dominant, such changes above mentioned were not apparent (see Saito et al., 2001; and Onuma et al., 2003).

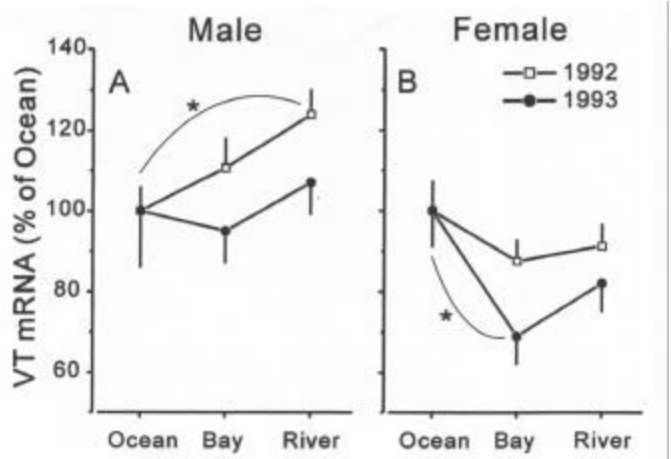


Fig. 1 Changes in the levels of vasotocin GH/PRL/SL mRNAs in homing salmon.

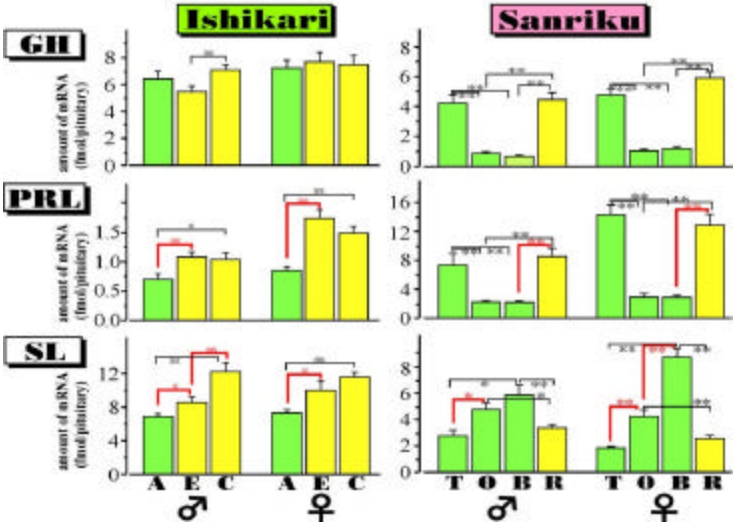


Fig. 2 Changes in the levels of pituitary mRNA in the Otsuchi stock chum salmon during homing migration (*, P < 0.05). Comparison of long and short rivers.

Similar tendency, the decrease in the amounts of vasotocin mRNA in females (Hiraoka et al., 1997) and increase in the amount of PRL in both sexes during upstream migration, were also seen in homing fish of Ishikari stock (Fig. 2), although influences of sexual maturation were not apparent probably because of temporal and spatial separation of FW entrance and final maturation in this long river stock.

The present results showed year-to-year differences in the pattern of changes in expression of several hormone genes including those for neurohypophysial hormones and prolactin, probably because of the variation of sea surface temperature (SST) which modulate upstream migration. High SST, which delay arrival to the natal river after sexual maturation, results in malfunction of hypo-osmotic adaptation.

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**ACTIVATION OF THE
GROWTH HORMONE/INSULIN-LIKE GROWTH FACTOR I AXIS
DURING SMOLTING AND SEAWATER ACCLIMATION
OF ATLANTIC SALMON, *Salmo salar*.**

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EXTENDED ABSTRACT ONLY - DO NOT CITE

The movement of fish between environments of differing salinities requires physiological adjustment of ion regulatory mechanisms that are under hormonal control. Classically, cortisol has been viewed as "the" seawater adapting hormone and prolactin as "the" fresh water adapting hormone. Recent evidence suggests, however, that other hormones are also critical for physiological acclimation of fish to fresh water and seawater (McCormick, 2001). In particular, growth hormone (GH) and insulin-like growth factor I (IGF-I) appear to be involved in the control of seawater acclimation in several euryhaline fish. There is, however, relatively little information on the regulation of growth hormone and especially insulin-like growth factor I following seawater exposure.

Coincident with their downstream migration, juvenile Atlantic salmon (*Salmo salar*) undergo preparatory physiological changes that are adaptive for seawater entry. Among the changes that occur during the parr-smolt transformation is an increase in salinity tolerance, allowing smolts to make rapid transitions into

seawater with minimal ionic and osmotic disturbance (Hoar 1988). Atlantic salmon also have the capacity to gradually acclimate to seawater over much of their life. Salmon are therefore an interesting and useful species to examine endocrine changes during preparatory changes and acclimation responses to increased salinity. In the present study we have examined changes in GH and IGF-I during smolting and after seawater exposure of parr. In addition, gill ornithine decarboxylase activity was measured as an index of gill mitotic activity to determine the relationship of this parameter with endocrine changes.

Methods

Experiment 1: Seawater Exposure of Parr

On December 15, Atlantic salmon (*Salmo salar*) parr were transferred from fresh water to 30 ppt seawater, and an identical number of fish were transferred to a fresh water control tank. Both groups were maintained at 10 °C, and fish were not fed during the experiment. Six fish per group were sampled prior to and 6 h, 1, 2, 3, 7 and 14 days after exposure to 30 ppt seawater.

Experimental 2: Changes during Parr-Smolt Transformation

Atlantic salmon juveniles were reared under natural photoperiod and temperature conditions. Beginning in January, 12 fish were sampled every month through June to examine changes that occur during smolting. Salinity tolerance was also examined monthly by exposing fish to 35 ppt seawater and measuring plasma osmolality after 24 hours.

In each experiment fish were anesthetized and bled from the caudal vessels, blood centrifuged and plasma stored at -80 °C for later analysis of hormones and/or ions and osmolality. Gill tissue was frozen at -80 °C and gill ornithine decarboxylase and Na,K-ATPase activity measured as outlined in Benfey (1992) and McCormick (1993), respectively. Plasma GH and IGF-I were measured by a fully validated radioimmunoassays as outlined in Björnsson et al. (1994) and Moriyama (1994), respectively. Plasma cortisol was measured by a fully validated enzyme immunoassay as outlined by Carey and McCormick (1998).

Results and Discussion

Plasma cortisol and GH increased after exposure of parr to seawater, but there was also high variability in both these parameters, perhaps reflective of

increased turnover of these hormones induced by seawater. Plasma IGF-I remained constant in the freshwater control group, whereas plasma IGF-I rose after 1 d in seawater and was significantly higher than in fresh water throughout the remainder of the study (Figure 1). Gill ornithine decarboxylase activity rose continuously after seawater exposure and was 10-fold higher than in fresh water at day 7 (Figure 1). At day 14 gill Na⁺, K⁺ ATPase activity was 4-fold higher in seawater than in fresh water (Figure 1).

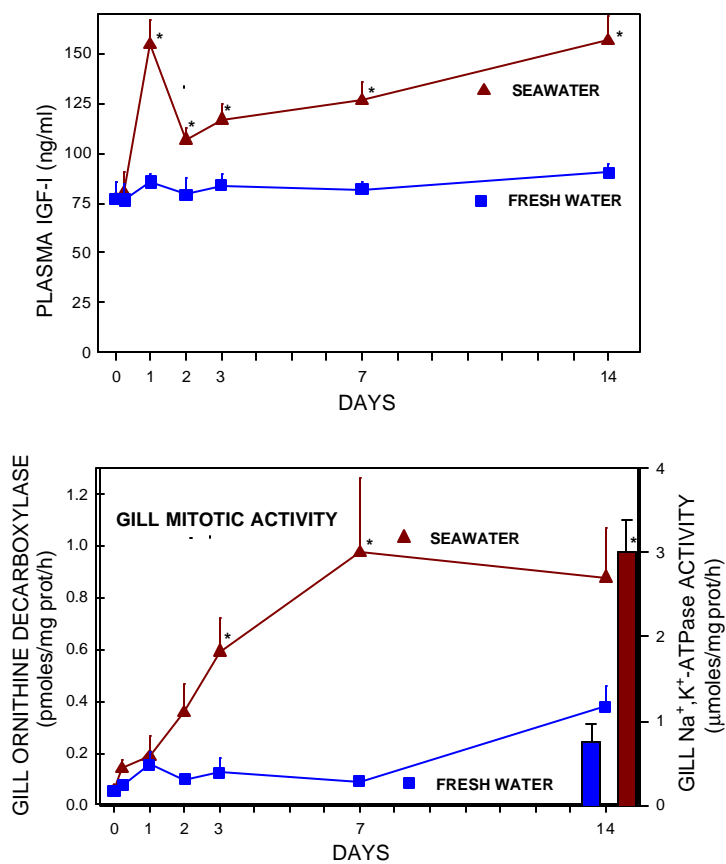


Fig. 1. The effect of salinity on plasma IGF-I, gill ornithine decarboxylase activity and gill Na⁺, K⁺ ATPase activity in Atlantic salmon parr. Fish were sampled prior to and 6 h, 1, 2, 3, 7 and 14 days after exposure to 30 ppt seawater. Control fish remained in fresh water under otherwise identical conditions to seawater-exposed fish. Values are mean \pm SE. An asterisk indicates a significant difference ($p < .05$, Newman-Keuls test) between fresh water and seawater groups at a given time point.

Smolts had a 3-fold increase in gill Na⁺, K⁺ ATPase activity and increased salinity tolerance between January and May followed by a decrease in June; there was a strong correlation between these two parameters ($r^2=0.87$). Gill ornithine decarboxylase remained relatively constant between Jan and May, then rose by 50% in June. Plasma cortisol was low in Jan and Feb (~3 ng/ml), rose to peak levels in April (20 ng/ml) and declined in June. Plasma GH was low from Jan-Mar (1-3 ng/ml), peaked in April (17 ng/ml), then declined in May and June. Plasma IGF-I was low in Jan (54 ng/ml), rose steadily through May (169 ng/ml), then declined in June. During smolting, peak levels of gill Na⁺, K⁺ ATPase activity and salinity tolerance coincided with peak plasma IGF-I levels, and were preceded one month by peak levels of cortisol and GH.

The results indicate that the GH/IGF-I axis is activated by both seawater acclimation and during the parr-smolt transformation of Atlantic salmon. Previous studies have demonstrated that exogenous GH and IGF-I can independently increase salinity tolerance (see McCormick 2001), and that these hormones interact positively with cortisol to increase seawater adaptability. In combination, these results provide compelling evidence that the GH/IGF-I axis is critical for the normal development of salt secretion in Atlantic salmon.

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**THE CONSEQUENCE OF FEEDING IN THE DOGFISH
SHARK FOR ACID-BASE REGULATION AND NITROGEN
METABOLISM**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Elasmobranch fish utilize nitrogenous waste products as osmolytes to maintain osmotic balance. Urea is the predominant osmolyte and is synthesized via the ornithine urea cycle (OUC) enzymes and glutamine synthetase (GSase), as glutamine is the required as the substrate for nitrogen donation to the OUC. Despite reabsorption of urea from the kidney tubules (e.g. Morgan et al. 2003) and a gill epithelium (e.g. Pärt et al. 1998, Fines et al. 2001) that is relatively impermeable to urea, urea is lost to the environment and must be replaced. Since synthesis of urea via the OUC is energetically expensive (Anderson 2001) and dogfish may feed only at irregular intervals, we hypothesised that dogfish would have an acid-base disturbance associated with feeding, as well as a strong N-conservation system and an effective N-conversion system from amino acids to urea. This study investigated the effect of feeding, and thus elevated nitrogen status in Pacific spiny dogfish (*Squalus acanthias*), food-deprived for 1 week.

Adult dogfish (about 2,000 g) were collected by trawl netting and were held in a 75,000 L circular tank at Bamfield Marine Station, and then were fed twice weekly with flatfish and herring. Both natural feeding and experimental feeding

by stomach tube were investigated. In the natural feeding, sharks were transported to a small tank (36 L) an hour after feeding with whole dead flatfish and herring in the circular tank. In the experimental feeding, sharks were collected from the circular tank, anaesthetised, and surgical implantation of a stomach cannula, and a caudal arterial blood catheter were performed. Experimental food consisted of equal weights of minced flat fish and saline, infused via the implanted stomach tube.

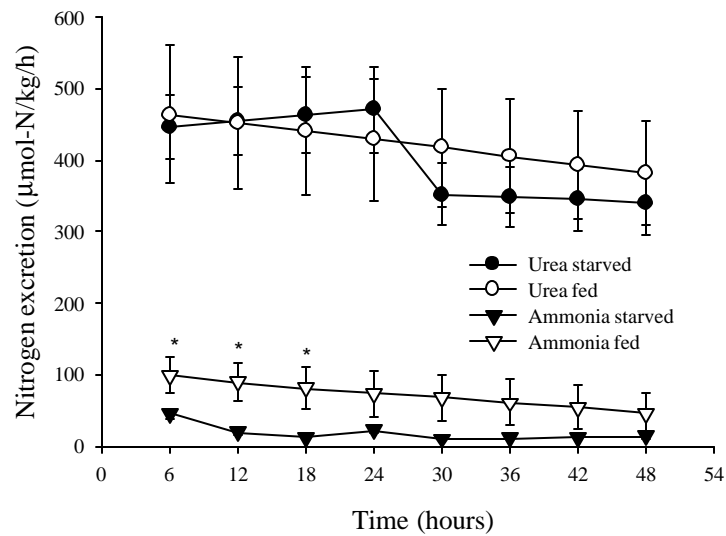


Figure 1. The effect of natural feeding on urea and ammonia excretion in *S. acanthias* over 48 hours following a feeding event. Data are presented as means \pm SEM. (starved N = 12; fed N = 8)

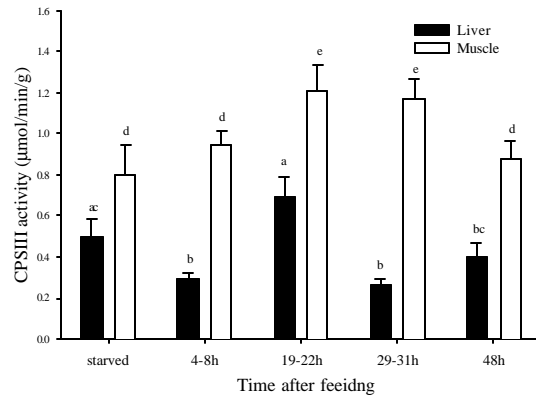


Figure 2. CPSase III activities in the liver and muscle of *S. acanthias* after a natural feeding. Data are presented as means \pm SEM. (N = 3-12)

A marked disturbance of blood acid-base status (“alkaline tide”) and a small rise in plasma ammonia followed experimental feeding. In both treatments, only minor increases in ammonia excretion and no change in urea excretion occurred, indicating strong N-retention after feeding (Fig. 1).

Experiments were performed to determine enzyme activities related to nitrogen and fat metabolism in liver and extrahepatic tissues. This study shows that carbamoyl phosphate synthetase III (the rate-limiting enzyme of OUC) activity in the muscle is higher compared to the liver and that the activities increase after feeding (Fig. 2). The contribution of muscle to urea synthesis in the fish body appears to be much larger than that of liver when body mass is considered. Furthermore, enhanced activities of nitrogen scavenging enzymes (e.g. GSase), arginase and enzymes of fat metabolism (e.g. B-hydroxybutyrate dehydrogenase) were seen after feeding in both liver and extrahepatic tissues. These changes were accompanied by a marked, persistent decline in plasma B-hydroxybutyrate and small delayed increases in plasma urea, osmolality and trimethylamine oxide. Urea excretion was unchanged, but ammonia excretion was raised in fed fish. In conclusion, we suggest that N-conservation is a high priority in this elasmobranch, and that feeding promotes ureogenesis and growth. Furthermore exogenous nitrogen from food is converted into urea not

only by the liver but by the whole body, while calories are directed into fat metabolism.

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Acknowledgements

Tammy MacDonald, Andrea Hunter and Chris Glover are thanked for their help with these studies. An NSERC Discovery Grant to CMW and an NSF grant to PJW supported this research.

**MECHANISMS OF BICARBONATE TRANSPORT
BY THE MARINE TELEOST INTESTINE**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Marine teleosts rely on drinking to replace osmotic fluid loss to their concentrated environment and active uptake of NaCl by the intestinal epithelium drives for fluid absorption (Smith 1930). Traditionally, this salt absorption by the intestinal epithelium is attributed to Na:Cl and Na:K:2Cl co-transporters in the apical membrane. However, studies have revealed that intestinal Cl⁻ absorption greatly exceeds Na⁺ absorption and that K⁺ absorption is limited. These observations suggest that apical Cl⁻/HCO₃⁻ exchange contributes significantly to active Cl⁻ uptake across the intestinal epithelium (Wilson et al., 2002; Grosell et al., 2004), in some cases by as much as 70%. This leads to the obvious questions of driving force for the anion exchange process and source of HCO₃⁻ for the apical exchange with Cl⁻. In the present study, we addressed these questions by first considering the potential contribution of extracellular CO₂ and HCO₃⁻ to luminal HCO₃⁻ secretion and secondly by considering the driving force for the active luminal secretion of HCO₃⁻.

Materials and Methods

Toadfish, *Opsanus beta* were caught in Florida bay and were maintained in the laboratory in flow-through seawater on a diet of frozen squid for a minimum of

7 days prior to experimentation. Fish were anesthetized, decapitated and the mid segment of the intestine was obtained by dissection and mounted in an Ussing chamber. Experiments were conducted under *in vivo* like conditions with saline composition mimicking extracellular fluids on the serosal side and intestinal fluids on the mucosal side. The luminal half-chamber contained a pH-combination electrode and a burette tip. Luminal pH was recorded continuously and pH was maintained at 7.800 by automated addition of HCl (0.005N) while the epithelium was held at current clamp conditions (0 μ Amp). Current pulses (50 μ Amp) of 2 sec duration every 60 sec allowed for simultaneous recordings of epithelial conductance. Electrophysiology and pH-stat titration data was logged to PC's continuously and epithelia were stable and viable for at least 8-10 hours under these conditions.

The possible contribution of serosal HCO₃⁻ and CO₂ to luminal HCO₃⁻ secretion was tested by measuring HCO₃⁻ secretion rates under conditions with HCO₃⁻ and CO₂ present in the serosal saline (5 mM and 0.3% in O₂, respectively) and with a HEPES buffered serosal saline gassed with 100 % O₂. Hydration of CO₂ may depend on the enzyme carbonic anhydrase, and yield not only HCO₃⁻ but also liberates a proton. Protons liberated from CO₂ hydration in the intestinal epithelium must be extruded from the epithelial cells in order to maintain intracellular pH and this proton extrusion must occur across the basolateral membrane since the epithelium perform net base secretion. The possible involvement of carbonic anhydrase (CA) in intestinal HCO₃⁻ secretion was tested by applying the CA blocker etoxzolamide (10⁻³-10⁻⁴ M) to the luminal saline following an initial control period.

Results and Discussion

Replacing serosal HCO₃⁻/CO₂ with HEPES/O₂ had limited if any effect on luminal HCO₃⁻ secretion (Fig. 1) and electrophysiological parameters which is in absolute agreement with our previous findings from the European flounder, *Plactichthys flesus* (Wilson & Grosell 2003). These findings demonstrate that endogenous, epithelial CO₂ is the source of HCO₃⁻ for luminal secretion and that this may be a

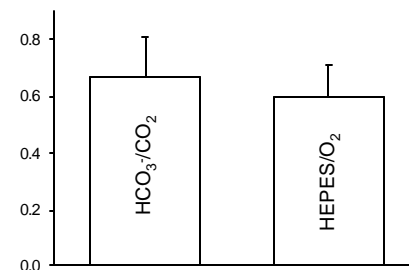


Fig. 1. HCO₃⁻ secretion (μmol cm⁻² h⁻¹) in *Opsanus beta* in presence and absence of serosal HCO₃⁻ and CO₂ (n=6).

general phenomenon for marine teleosts since it applies for the two species tested to date.

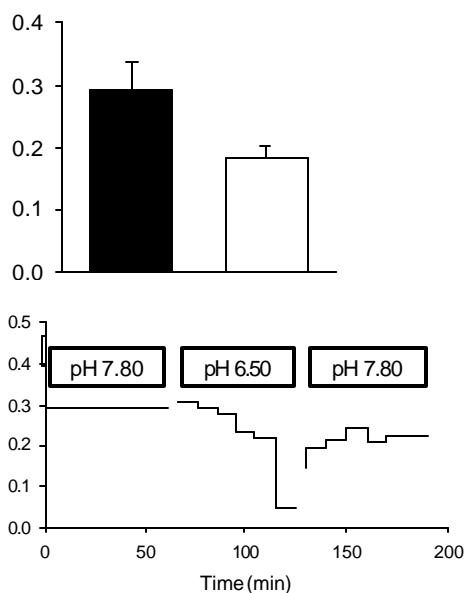


Fig. 2. Top panel: Effect of etoxzolamide (10^{-3} M) (open bar) on luminal HCO_3^- secretion ($\mu\text{mol cm}^{-2} \text{h}^{-1}$). Dark bar represents control, $n=4$ in both cases. Bottom panel: Effect of serosal acidification on luminal HCO_3^- secretion ($\mu\text{mol cm}^{-2} \text{h}^{-1}$). Data from a single representative preparation is displayed.

It appears that CA mediated hydration of cellular CO_2 is of some importance for luminal HCO_3^- secretion since treatment with the lipophilic CA inhibitor etoxzolamide caused an approximately 40% reduction in HCO_3^- secretion (Fig. 2, top panel). This incomplete inhibition was observed despite the addition of high concentrations of etoxzolamide. Addition of 10^{-4} and 10^{-3} M resulted in similar degree of inhibition suggesting that maximal inhibition of CA was achieved by 10^{-3} M etoxzolamide.

These observations suggest that non-catalyzed CO_2 hydration is sufficient to sustain a substantial part of the CO_2 hydration required to fuel the apical HCO_3^- secretion.

Intestinal HCO_3^- secretion represents active transport (Grosell et al., 2004) and is mediated by apical $\text{HCO}_3^-/\text{Cl}^-$ exchange (Grosell et al.,

2001). However, apical anion exchange cannot alone explain the apparent active transport which is hypothesized to depend on active extrusion of protons across the basolateral membrane. To test this hypothesis experiments were performed with reduced serosal pH to reduce or inhibit basolateral proton extrusion. These experiments, as predicted, resulted in an initial reduction and subsequently complete inhibition of luminal HCO_3^- secretion, an effect which was reversible (Fig. 2 bottom panel).

Ongoing studies are investigating the nature of the basolateral proton extrusion which appears to be fueling the active secretion of HCO_3^- across the apical membrane.

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**MULTIPLE ROLES OF INTESTINAL BICARBONATE SECRETION
IN MARINE TELEOSTS**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Marine teleosts live in a hyper-osmotic, high-calcium and high-magnesium environment. To avoid dehydration they drink large quantities of the external medium and must extract sufficient free water from the imbibed fluid to maintain osmotic balance, whilst ideally minimising the absorption of divalent ions. The established model describing the mechanism of intestinal water absorption involves the active uptake of Na^+ and Cl^- ions first (via apical Na:Cl and Na:K:2Cl co-transporters) which drives the net uptake of water. However, more recently it has been established that a substantial fraction of the Cl^- is absorbed via apical Cl/HCO_3^- exchange within the intestine (Grosell et al., 2001; Wilson et al., 2002). The secreted bicarbonate is derived from cellular CO_2 , which as a gas has no osmotic influence, and so Cl/HCO_3^- exchange can effectively drive water uptake as it represents the net movement of osmolytes (i.e. chloride) into the cell from the intestinal lumen (Wilson et al., 2002; Wilson & Grosell, 2003). Thus, the secretion of bicarbonate plays an important role in osmotic regulation by being directly linked to intestinal water absorption.

The net secretion of bicarbonate via this mechanism also plays a second, more indirect role in osmotic regulation, by causing the accumulation of very high concentrations of HCO_3^- within the luminal fluid (e.g. 40-130 mM; Wilson et al., 1996) as well as a relatively high pH (8.4-9.0; Wilson, 1999). This promotes the precipitation of imbibed Ca^{2+} and Mg^{2+} as their insoluble carbonates. We have presented a novel mechanism of intestinal water transport (Wilson, 1999; Wilson et al., 2002; Wilson & Grosell, 2003) whereby net secretion of HCO_3^-

paradoxically facilitates the net absorption of water, because the precipitation and excretion of Ca and Mg effectively removes osmolytes from the gut lumen. This enhances the absorptive osmotic gradient and allows marine teleosts to maintain a lower plasma osmolality than would arise without this precipitation (Wilson et al., 2002).

A second important physiological effect of intestinal bicarbonate secretion is that precipitated Ca and Mg are excreted as solids, rather than being absorbed. This is particularly important in the marine teleosts whose kidneys are the primary site for excretion of excess divalent ions and yet have exceptionally low urine flow rates, leading to the constant risk of renal stone formation (primarily CaCO₃). The absorption of 85% of the imbibed water volume along the intestine (as occurs in many teleosts; Wilson, 1999) would result in an accumulation of calcium to concentrations as high as 70 mM and hence an increased driving force for absorption into the blood. However, the actual calcium concentration within the intestinal fluid of marine teleosts is kept much lower, usually < 5 mM, almost entirely due to its precipitation and excretion as solid CaCO₃ (Wilson & Grosell, 2003), and therefore minimising the risk of renal stones. Intestinal bicarbonate secretion therefore plays a rather novel role in calcium homeostasis in marine teleosts, by limiting their entry into the body fluids from the Ca/Mg-enriched environment they live in.

The excretion of both bicarbonate-rich rectal fluid and carbonate precipitates represents a significant loss of base that is anatomically separated from the uptake/excretion of other acid-base relevant ions that primarily occurs at the gills in marine teleosts (Wilson et al., 1996). Thus intestinal bicarbonate secretion plays a third role in the acid-base balance of marine teleosts, although it does not appear to be regulated in response to acid-base disturbances (e.g. metabolic alkalosis resulting from vascular infusion of NaHCO₃; Wilson et al., unpublished data). However, conversely, other factors that can alter the rate of intestinal bicarbonate secretion do cause acid-base disturbances in the predicted direction. For example, intestinal bicarbonate secretion is specifically stimulated by the presence of Ca²⁺ ions with the gut fluid in European flounder (*Platichthys flesus*; Wilson et al., 2002). Furthermore, adding extra calcium (70 mM) to sea water (and hence imbibed fluid) results in a 40% depletion of plasma total CO₂ in flounder (Wilson et al., 2003) and reducing calcium in artificial seawater being imbibed by the Gulf toadfish (*Opsanus beta*) causes a significant 42% elevation in plasma total CO₂ and an alkalosis (Table 1).

Table 1. Plasma pH and total CO₂ concentration in Gulf toadfish following 3 days exposure either low (0.5 mM) or normal (10 mM) calcium in artificial seawater. Values shown are means ± SEM (N). Asterisks represent significantly different from control fish at P<0.05 (*) or P < 0.01 (**).

	<u>Plasma TCO₂ (mM)</u>	<u>Plasma pH</u>
Control Seawater	6.87 ± 1.29 (7)	8.00 ± 0.14 (4)
Low Ca ²⁺ Seawater	9.79 ± 1.48 (7)**	8.16 ± 0.12 (6)*

Therefore, changes in intestinal bicarbonate can have a significant impact upon whole animal acid-base status. The precise mechanism of how calcium regulates intestinal bicarbonate secretion is yet to be elucidated, but the multiple physiological roles it plays make this an intriguing area for future study.

Acknowledgements

The data reported was supported by a Royal Society grant (RSRG 24241 to RWW), and a RSMAS NIEHS Pilot Project grant (to MG).

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ACID-BASE REGULATION IN AMAZONIAN FISHES

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EXTENDED ABSTRACT ONLY- DO NOT CITE

Introduction

Environmental hypercapnia occurs routinely in aquatic environments. It is most pronounced in tropical waters covered with dense mats of vegetation where CO₂ tensions have been reported to rise in excess of 60 mmHg (Heisler et al., 1982). In freshwater, hypercapnia induces a respiratory acidosis in fish that is compensated within 24-96 h. Plasma pH (pHe) compensation is associated with accumulation of plasma HCO₃⁻ (in exchange for Cl⁻), predominantly at the gills, with the kidney playing a relatively minor role (Heisler, 1984). The rate and degree of pHe recovery following exposure to hypercapnia is highly dependent upon water ionic composition (Larsen and Jensen, 1997). Given that Amazon waters are characteristically low in ions (approaching that of distilled water), endemic fishes may be compromised in their ability to regulate acid-base status; however, this has received limited attention.

The armoured catfish, *Liposarcus pardalis*, a teleost from the Amazon, tolerates large fluctuations in water oxygen and carbon dioxide tensions and may be routinely exposed to bouts of environmental hypercapnia in the natural

environment. In a previous study, *L. pardalis* did not regulate pHe during exposure to environmental hypercapnia (PCO₂ of 40 mmHg), permitting blood pH to fall by up to 1 pH (Brauner et al., 2002). Most vertebrates completely or at least partially regulate pHe during hypercapnia (Heisler, 1984); however this is clearly not the case in *L. pardalis*. The purpose of this study was to investigate the effect of incomplete pHe regulation during environmental hypercapnia on pHi regulation of the heart, liver and white muscle in *L. pardalis* to gain insight into the mechanisms through which this species tolerates exposure to hypercapnia.

Materials and Methods

Liposarcus pardalis, (308 ± 15.5g) were obtained from a commercial fish supplier and held at the National Institute for Research of the Amazon (INPA), Manaus, Brazil, for at least 2 weeks prior to experimentation. Fish were placed in individual 2 L chambers over-night and then exposed to a PCO₂ of 14 or 32 mmHg without access to air. At 0, 6, 24 or 72 h following exposure to hypercapnia, fish were anaesthetized with a concentrated solution of buffered MS-222. Within 2-3 minutes, fish lost equilibrium and could be removed from the water. Using a heparinized syringe, blood was drawn from the caudal vein for measurement of pHe, red cell pHi, PCO₂, plasma ion levels (Na⁺, Cl⁻, Ca²⁺ concentrations) and osmolarity. Fish were euthanized and 0.5g of heart, liver and white muscle were removed and stored in liquid nitrogen for later analysis of pHi. Sampling was complete within 2-3 mins after the fish had been removed from the water. In a parallel study, whole animal unidirectional Na⁺ influx (J_{in}Na⁺), efflux (J_{out}Na⁺), net flux (J_{net}Na⁺), net Cl⁻ flux (J_{net}Cl⁻), and total ammonia excretion were measured at 0, 6 and 24h following exposure to a PCO₂ of 32 mmHg.

Results and Discussion

During exposure to hypercapnia (1, 2 and 6% CO₂), *L. pardalis* exhibit a pronounced extracellular acidosis that, for 24-96 h, remains largely uncompensated. In most vertebrates, pHi is greatly influenced by pHe and the time course of pHi recovery is similar to that for pHe recovery. Despite a large reduction in pHe, heart, liver and white muscle pHi was regulated within 6 h (the earliest time point at which tissue pHi was measured) through accumulation of intracellular HCO₃⁻. Only two other vertebrates have been reported to be

capable of complete preferential regulation of pH_i in the face of a largely uncompensated extracellular acidosis as observed in *L. pardalis*. When the salamander (*Siren lacertian*) is exposed to aquatic hypercapnia ($PCO_2 = 35$ mmHg) with access to normoxic air (Heisler et al., 1982), or when the facultative air-breathing fish *Synbranchus marmoratus*, is forced to breath air during exposure to aquatic hypoxia, both vertebrates exhibit a large uncompensated respiratory acidosis but completely regulate heart and white muscle pH_i by 72 or 24 h respectively (the only time periods measured in those studies; Heisler, 1982; Heisler et al., 1982).

The magnitude of the extracellular acidosis tolerated by *L. pardalis*, and the ability to regulate pH_i in the face of an extracellular acidosis are the greatest reported for a teleost fish. It is not known whether this pattern of acid-base regulation is associated with the ability to breathe air, with living in water low in counter ions, or with other evolutionary or environmental pressures. The ubiquity of this strategy among Amazonian fishes and the mechanisms employed remain to be investigated.

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Acknowledgements

This study was supported by NSERC Canada Discovery Grants to CJB, YW and NJB, NSERC and Killam Post-doctoral fellowship to JR, University Faculty fellowship to RG, and CNPq Brazil research grant to ALV. TW was supported by The Danish Research Council. We thank Aline Matsuo and Nazare Paula da Silva for excellent technical assistance.

**PHYSIOLOGICAL IMPACTS OF SHORT-TERM,
SUB-LETHAL ACID AND ALUMINUM EXPOSURE
OF ATLANTIC SALMON, *Salmo salar*, SMOLTS**

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Anthropogenic acidification has been implicated as a major factor in the disappearance of Atlantic salmon from rivers of eastern North America and Northern Europe. Episodic acid pulses during spring snowmelts and fall storms decrease surface water pH and increase the mobilization of aluminum (Al) from the soil (Driscoll and Schecher, 1988). Resulting elevated concentrations of dissolved Al, specifically, inorganic monomeric Al or labile Al, have toxic effects on fish, the severity of which may be altered by ambient calcium and dissolved organic carbon (Gensemer and Playle, 1999).

In fish, the classic toxic effect of increased dissolved Al and decreased pH is ionoregulatory disturbance (McDonald et al., 1991). The Atlantic salmon smolt or downstream migratory stage is the most sensitive of the salmon life history stages to ionoregulatory disturbances resulting from acid/Al exposure (Rosseland et al., 2001). Smolts may be particularly vulnerable to such disturbances as a result of their need to develop a high degree of seawater tolerance that will allow them to rapidly enter seawater with minimal ionic and osmotic disturbance.

In this study, we examined the physiological impacts of short-term, sub-lethal acid/Al exposure of Atlantic salmon smolts. Our objectives were two-fold; one to examine acid/Al impacts on smolt seawater tolerance and endocrinology under controlled laboratory conditions, and two, to examine possible mechanisms underlying these impacts.

Methods

Atlantic salmon smolts were randomly assigned to replicate tanks receiving either control (pH 6.5-7.0) or acid/Al conditions (pH 5.5-6.0, 200 µg/l total Al). Artificial softwater for all tanks was prepared by mixing deionized water with ambient river water. Acid/Al conditions were created by the addition of HCl and $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ to artificial softwater in header tanks, and water was mixed for >1h before entering experimental tanks to avoid highly toxic, unstable water conditions. Daily pH measurements were made directly in the tank, and water samples were taken for the measurement of total aluminum, labile aluminum, and calcium.

Experiment #1

Smolts were exposed to control and acid/Al conditions for 48 and 120 h. At each time point, 5 fish/tank were sampled and 5 fish/tank were placed in 30 ppt seawater. Fish in seawater were sampled 24 h later.

Experiment #2

Smolts were exposed to control and acid/Al conditions for 48 h and 120 h. At each time-point, 5 fish/tank were sampled and 5 fish/tank received an intraperitoneal injection of 0.3mg/g bromodeoxyuridine (BrdU) (a cell proliferation marker). Injected fish were returned to their respective tanks and then sampled 12 h later. Three injected fish/tank were sampled 3 days later.

All sampled smolts were anesthetized, length measured and weighed. Gill biopsies were taken for the measurement of aluminum content and Na^+ , K^+ ATPase activity. Gill tissue was also taken for immunocytochemistry and western immunoblotting. Blood was collected for the measurement of hematocrit, plasma ions, and plasma hormones.

Results and Discussion

Experiment #1

Short-term, sub-lethal acid/Al exposure had no impact on freshwater ionoregulatory ability, however it significantly compromised seawater tolerance as indicated by decreased gill Na^+ , K^+ ATPase activity (Fig. 1) and increased plasma chloride in seawater.

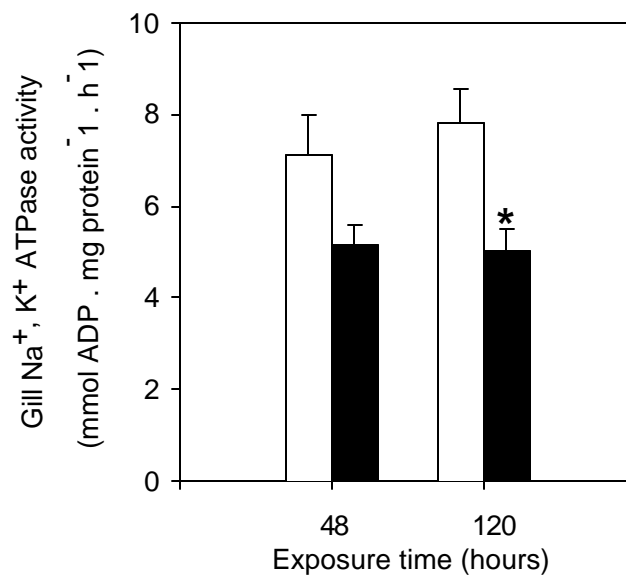


Fig. 1. The effect of 48 and 120 h of acid/Al exposure on gill Na^+ , K^+ ATPase activity. White bars indicate control groups and black bars indicate treatment groups. An asterisk indicates a significant difference ($p < .05$) between control and treatment groups within a time-point.

Acid/Al exposure had no effect on plasma cortisol, thyroid hormones, growth hormone and IGF-1, however it did elicit a significant stress response as indicated by increased hematocrit and plasma glucose. We suggest that acid/Al exposure is compromising seawater tolerance by negatively impacting gill ion

transport mechanisms, and that these effects on smolt ion regulation may only be detectable after seawater entry.

Experiment #2

Acid/Al exposure caused a 30% reduction in gill Na⁺, K⁺ ATPase activity at 120 h, however differences in gill Na⁺, K⁺ ATPase protein were undetectable as measured by western immunoblotting. Exposure to acid/Al for 120 h resulted in an increase in hematocrit and a slight decrease in plasma chloride. There was a slight, but significant increase in gill cell proliferation (# of BrdU-positive cells) after 120 h of acid/Al exposure, with the majority of the staining located on primary filaments and little to no staining on secondary lamellae. In both treatment and control groups, BrdU-labeled cells were not identified as chloride cells (Na⁺, K⁺ ATPase-positive) within 3 days following BrdU injection. Our results suggest that short-term, sub-lethal acid/Al exposure may be compromising seawater tolerance by decreasing gill Na⁺, K⁺ ATPase, however, this effect may be due to the direct inhibition of the enzyme by Al rather than to a change in the amount of gill Na⁺, K⁺ ATPase protein. Our results also indicate that acid/Al exposure causes an increase in gill cell proliferation, but over the time-course of 3 days these newly differentiated cells do not become mature chloride cells. The continuation of this study involves the examination of gill chloride cell necrosis and apoptosis, and the measurement of direct effects of Al on the activity of Na⁺, K⁺ ATPase.

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**BRANCHIAL ACID-BASE REGULATION
IN THE DOGFISH, *SQUALUS ACANTHIAS***

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

In marine Elasmobranches, the gills are the major site of acid-base regulation (Heisler, 1988). In this epithelium, apical sodium-proton exchange likely takes place via NHE2 and/or 3 (Edwards et al, 2002), whereas the only chloride-bicarbonate exchanger identified in elasmobranches so far is an apical Pendrin-like protein (Piermarini et al, 2002). In those studies, NHEs and Pendrin co-localized in the same cells with Na^+/K^+ -ATPase and H^+ -ATPase, respectively. The aim of this study is to determine the acid-base regulatory mechanisms in the gills of the dogfish, *Squalus acanthias*. Specifically, the importance of NHE2, Na^+/K^+ -ATPase and H^+ -ATPase in alkalotic and acidotic fishes was investigated.

Material and methods

Pacific spiny dogfish (*Squalus acanthias*) were obtained from commercial fishermen, and held in a tank provided with flowing seawater (11°C, 31 ppt salinity) at the Bamfield Marine Sciences Center (British Columbia). 28 animals were used for this study ($2,33 \pm 0,36$ kg).

In order to induce an alkalosis or acidosis, fish were infused intravenously with either 125 mM or 250 mM NaHCO₃ (4.00 ± 0.70 mL h⁻¹ kg⁻¹). NaCl-infused fish and sham-operated fish served as controls.

Blood samples were taken during the infusion period, and pH, total CO₂, [Na⁺], [Cl⁻] and hematocrit % were measured by standard techniques. After 24 h of infusion, gill samples were snap frozen in liquid nitrogen for western blot and ATPase analyses. The ATPase assay described by McCormick (1993) was modified to determine both the ouabain (Na⁺/K⁺-ATPase inhibitor, 500 μM) and bafilomicyn (H⁺-ATPase inhibitor, 50 nM) sensitive ATPase activities in gill samples.

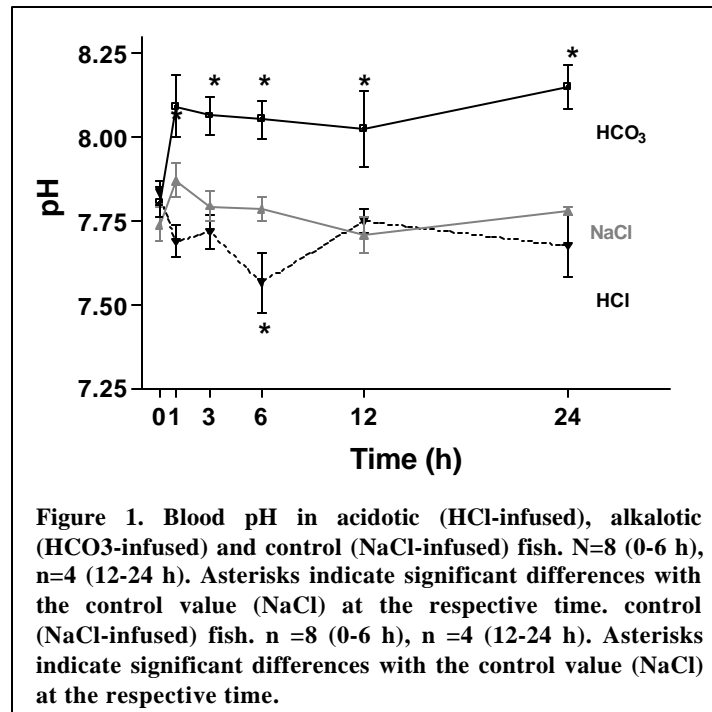
For Western blot analyses, 30 μL of total gill membrane protein were separated in 7.5 % polyacrylamide mini-gel (1 h at 180 V) and transferred to a nitrocellulose membrane. Binding of the primary antibodies (rabbit anti NHE2) was detected with a donkey anti-rabbit fluorescent secondary antibody. The protein bands were visualized and quantified using the Odyssey infra-red imaging system and software (Li-Cor Inc., USA).

Results were analyzed using 1-way ANOVA, 2-way ANOVA or RM-ANOVA when appropriate. Dunnet test (vs. sham-operated) was used for post-test comparisons in some cases.

Results and Discussion

The blood pH of base-infused fish (BIF) increased significantly after 1 h of infusion (from 7.8 ± 0.05 to 8.1 ± 0.05 pH units), and remained elevated for the rest of the experiment. The blood pH of acid-infused fish (AIF) dropped significantly after 6 h of infusion (7.55 ± 0.09 pH units), but it recovered to control values by time = 12 h (Figure 1).

Total CO₂ only changed significantly in the BIF. It increased from 4.15 ± 0.4 mM to 10.2 ± 1.15 mM after 1 h, and remained elevated for the rest of the infusion period. In AF and control fish, total CO₂ presented values between 2.1 and 4.4 mM.



The other blood parameters did not show any significant difference between the treatments. ([Na⁺]-248 mM; [Cl⁻]-268 mM; osmolarity ~966.5 mM; hematocrit~90% of initial value).

The responses of NHE2, Na⁺/K⁺-ATPase and H⁺-ATPase are summarized in Table 1. The gills of AIF showed a 113% increase in NHE2-like expression. This suggests that the branchial epithelium compensates the blood acidosis by exchanging protons for Na⁺ from the seawater. The inward directed sodium gradient, along with the action of the Na⁺/K⁺-ATPase, would be sufficient to

drive electroneutral exchange through a NHE2-like protein, although involvement of NHE3-like cannot be excluded. The unchanged Na^+/K^+ -ATPase compared to the controls doesn't exclude a modulation of the *in situ* activity of the ATPase due to changes in intracellular Na^+ .

The blood pH of BIF did not recover to control values. However, the increase in H^+ -ATPase would only make sense if the protons were secreted into the blood, thus tending to counteract the alkalotic state. Similar responses to acidosis (Lucioni *et al*, 2002) and alkalosis (Verlander *et al*, 1992) have been found in the rat intestine and kidney, respectively.

Table 1. Ion-transporting proteins in gills of acidotic, alkalotic and control dogfish.

Infusion	NHE2-like relative abundance (A.b.s.)	Na^+/K^+ -ATPase (nmol ADP \cdot g protein ⁻¹ h ⁻¹)	H^+ -ATPase (nmol ADP \cdot g protein ⁻¹ h ⁻¹)
HCl (24 h)	213 \pm 34*	2.5 \pm 0.6	0.03 \pm 0.02
HCO_3^- (24 h)	97 \pm 4	4.9 \pm 2.0	1.04 \pm 0.33*
NaCl (24 h)	74 \pm 7	2.7 \pm 1.2	0.21 \pm 0.08
Sham-operated	100 \pm 21	1.1 \pm 0.5	0.03 \pm 0.03

n = 4 for all groups

A.b.s. = arbitrary densitometric units, from Western blots.

* indicates a significant difference with the Sham-operated (1-way ANOVA - Dunnet test).

In conclusion, our results confirm the involvement of the gills in acid-base regulation in marine Elasmobranchs. The cellular mechanism would include apical Na^+/H^+ exchange through an apical NHE2-like protein, whose expression is increased in acid-infused fishes. H^+ -ATPase seems to be involved in the response to alkalosis, most likely introducing protons into the blood.

Future research will include immunohistochemical studies in order to determine the response of the gill cells to the acid- and base-infusions. Specifically, we'll look for differences in the number and/or localization of Na^+/K^+ -ATPase-rich cells and H^+ -ATPase-rich cells within the gill filament. Quantification of the

relative abundance of a Pendrin-like protein in the different conditions will also be performed.

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Acknowledgements

Supported by an NSERC grant to GGG, the Donald Ross Scholarship to MT, the Department of Biological Sciences at the University of Alberta, and the Bamfield Marine Sciences Station. FK receives support from The Japan Society for the Promotion of Science for Young Scientists.

**CALCIUM UPTAKE IN TWO AMAZONIAN FISH
NATIVE TO ION-POOR WATERS
OF THE RIO NEGRO**

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Introduction

Calcium (Ca^{2+}) is essential in a number of physiological processes such as the control of membrane permeability, activation of muscular contraction, transmission of nervous impulses, and bone formation. Freshwater fish are exposed to a constant supply of Ca^{2+} in the water, so uptake occurs predominantly through the gills. Uptake through the skin is negligible, but the intestine may participate under some conditions. Calcium uptake in Amazonian fish is poorly known. Only limited information on unidirectional Ca^{2+} flux is available for freshwater stingrays inhabiting extremely soft waters (Wood *et al.*, 2002). Indirect evidence, based on high acid tolerance, suggests that Ca^{2+} affinity is very high in some species from the Rio Negro basin.

We examined the kinetics of Ca^{2+} uptake in two Amazonian species (*Apistogramma* sp. and *Carnegiella strigata*) under both natural (on-site) and artificial conditions. On-site experiments involved testing blackwater from the Rio Negro, which has a high content of dissolved organic matter (DOM). Laboratory experiments were based on water with ion concentrations comparable to those of the Rio Negro, but without DOM (reference water). We

also investigated changes in Ca^{2+} uptake over time in fish exposed to lower pH and a higher concentration of DOM.

Materials and Methods

Calcium kinetics

On-site experiments used adult *Apistogramma* sp. and *Carnegiella strigata* collected from a tributary of the Rio Negro where water composition was: $\text{Na}^+=108$, $\text{Cl}^-=123$, $\text{Ca}^{2+}=10$, $\text{Mg}^{2+}=8$, $\text{K}^+=14$ (all μM); pH 3.9; DOM=22 mgC/l; 30°C. Kinetics was based on whole body ^{45}Ca uptake, following Perry & Wood (1985). J_{max} and K_{m} were obtained using a non-linear curve-fitting program.

Calcium kinetics was also examined under artificial conditions using wild-caught individuals of the same species, acclimated to the soft water with negligible DOM (reference water; $\text{Na}^+=34$, $\text{Cl}^-=28$, $\text{Ca}^{2+}=11$, $\text{Mg}^{2+}=0.8$, $\text{K}^+=15$ (all μM); pH 6.3; DOM=0.9 mgC/l; 28°C). Kinetics was performed as discussed above.

Effects of increasing DOM concentration and lowering pH

Apistogramma sp. and *Carnegiella strigata* collected on-site were used to assess changes in Ca^{2+} uptake resulting from experimentally lowering pH and increasing DOM concentration in the water. Treatments were as follows: a) control (Rio Negro water, composition reported above), b) AHA (37 mgC/l), c) low pH (3.6), d) AHA+low pH. The pH was lowered using HNO_3 , and DOM concentration was raised by adding Aldrich humic acid (AHA) to the Rio Negro water (initially at 22 mgC/l). Calcium influx over time was based on whole body ^{45}Ca uptake.

Results and Discussion

Calcium kinetics

Kinetics studies revealed a very high Ca^{2+} affinity for both *Apistogramma* sp. and *C. strigata* ($K_{\text{m}}\sim 27 \mu\text{M}$) tested in blackwater from the Rio Negro (a low K_{m} value indicates a higher affinity). Acclimation to reference water, however, resulted in a significant reduction in K_{m} ($K_{\text{m}}\sim 68 \mu\text{M}$). Likewise, the Ca^{2+} transport capacity (J_{max}) in fish tested on-site was much higher than in fish tested in reference water (Fig.1). Upon acclimation to reference water, J_{max} was reduced by 74% in *Apistogramma* sp. and by 50% in *C. strigata*, relative to

natural conditions. The most striking difference between the waters tested was the presence of high DOM concentrations in the Rio Negro water. One would expect that with elevated concentrations of DOM in the Rio Negro most Ca^{2+} in the water would be bound up to DOM, so virtually no Ca^{2+} would be available for uptake through the gills. Our results indicated, however, normal Ca^{2+} uptake rates in fish tested on-site. DOM is likely to play a role in ion transport as initially speculated by Gonzalez *et al.* (1998), but the mechanism remains unknown.

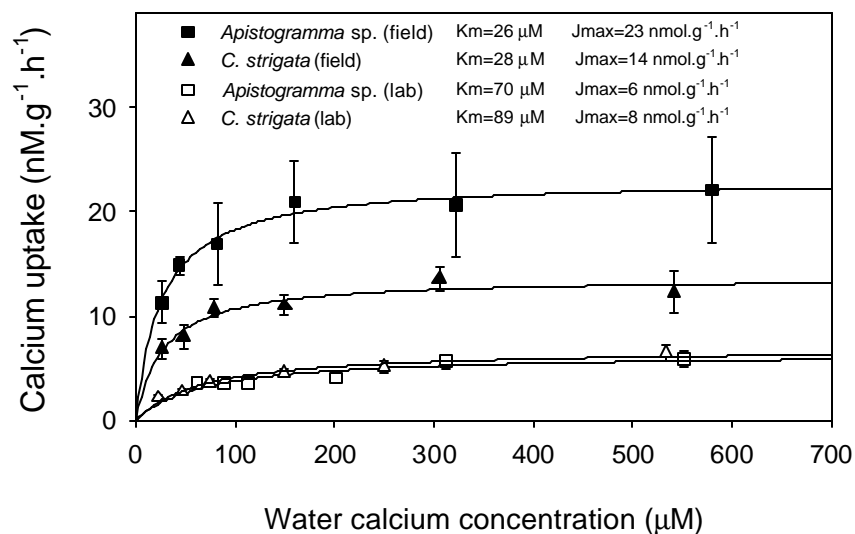


Figure.1. Kinetics of Ca^{2+} uptake in two species from the Rio Negro basin, with estimates of affinity (K_m) and transport capacity (J_{max}) for fish tested in natural blackwater ('field') and in reference water ('lab').

Calcium uptake under various treatments

Lowering the pH to 3.6 resulted in an inhibition of Ca^{2+} uptake by 80% in *Apistogramma* sp. and by 56% in *C. strigata* (Fig.2). Inhibition of Ca^{2+} uptake by low pH is believed to result from competition between Ca^{2+} and H^+ for gill binding sites. Because the Ca^{2+} concentration in the testing water was only $10\mu\text{M}$, the increased levels of H^+ probably out-competed Ca^{2+} at the uptake sites, thus inhibiting Ca^{2+} influx.

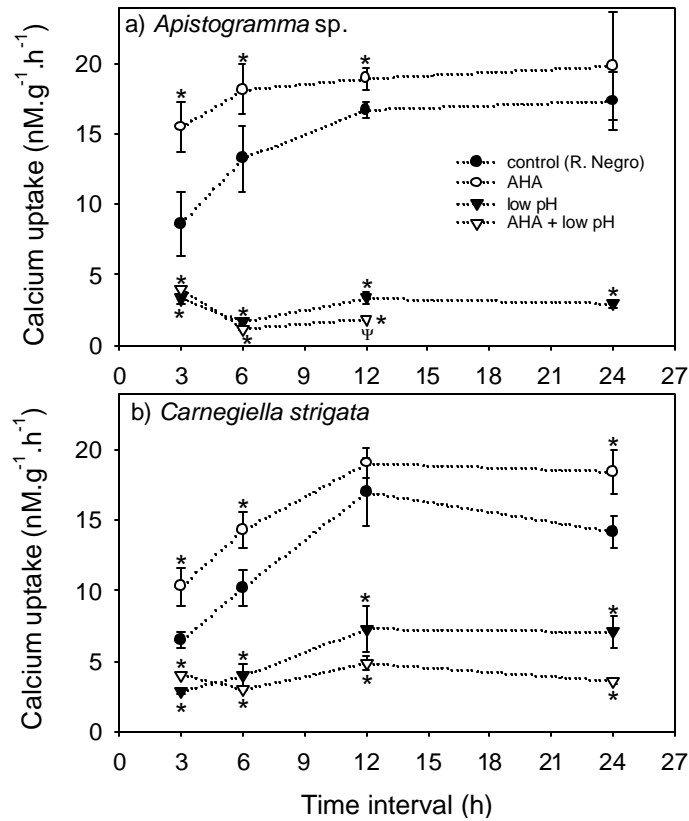


Figure.2. Effects of increasing DOM concentration and lowering the pH on Ca^{2+} uptake in two species from the Rio Negro. (*) indicates significant difference relative to control; (Ψ) indicates total mortality at the sampling time.

Increasing DOM concentration in the water by adding AHA resulted in stimulation of Ca^{2+} uptake by 30% in both species, suggesting that DOM stimulates Ca^{2+} transport capacity. It is also possible that this increased uptake capacity was reflecting increased Ca^{2+} loss due to increased membrane permeability. We were not able to measure Ca^{2+} efflux in this study. The

combination of AHA+low pH did not stimulate Ca^{2+} uptake in fish, probably because the buffering capacity of the natural blackwater had been exceeded.

Acknowledgements

This work was supported by the Potamotrygon Project (ACEPOAM), Turkey's Aquarium, INPA and CNPq (CTPetro). We are indebted to Carlos Pinheiro Castelo Branco for fish sampling and to Rosinalda das Chagas Teixeira for technical assistance in the field. AYOM was a recipient of a fellowship from CNPq (GD#141090/1999-5).

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**CHLORIDE LEVELS
IN THE GASTROINTESTINAL CONTENT
OF THREE TELEOST SPECIES
WITH DIFFERENT FEEDING HABITS**

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Interpretations of feeding habit based only on the items of the gastrointestinal content can lead to erroneous conclusions because these items may be related to their availability in the environment and not due to the fish preference (Barbieri et al., 1994). Consequently, a study of the morphology of the gastrointestinal tract is also needed. The intestine of freshwater teleosts is important for osmoregulation, but intestinal absorption is not completely understood because most studies were done with starved specimens (Baldisserotto, 2003), and feeding changes ionic situation of rainbow trout intestine (Dabrowski et al., 1986). Therefore, the objective of the present work was to analyze some aspects

of the morphology and chloride levels in the fluids of the gastrointestinal tract of three species of teleosts fish with different feeding habitats.

Methodology

Specimens of *Hoplosternum littorale* (omnivore, with preference for invertebrates) (n=6), *Rhamdia quelen* (omnivore, with preference for fish) (n=4), and *Hoplias malabaricus* (piscivore) (n=7) were collected with nets from ponds around the campus of the Universidade Federal de Santa Maria, in Southern Brazil. Fishes were transported to the Fish Physiology Laboratory and kept in aerated 250 L tanks for at most 2 h. Blood was collected from the caudal vein with heparinized 1-mL syringes, and centrifuged at 5,000 rpm for 5 min to separate plasma, which was stored at -20°C for analysis. Specimens were then sacrificed, weighed, measured, and digestive tract was dissected in stomach, anterior intestine (or pyloric ceca for *H. malabaricus*), medium (except *H. littorale*) and posterior intestine. The contents of these segments were collected separately and centrifuged at 7,000 rpm for 5 min to separate the fluid phase. The supernatant was stored in eppendorf tubes at -20°C for analysis. Intestinal quotient (IQ) was obtained by the equation: $IQ = \text{intestine length} / \text{body length}$. Digestive tract and liver were also weighed for determination of digestive somatic index ($DSI = (\text{digestive tract weight} / \text{body weight}) \times 100$) and hepatic somatic index ($HSI = (\text{liver weight} / \text{body weight}) \times 100$), respectively. Chloride concentration was measured according to Zall et al. (1956). Comparisons of parameters among the different species were made by one-way analysis of variance and Tukey test with the aid of the software Statistica 5.0. Minimum significance level was 95% ($P < 0.05$).

Results

The intestinal quotient is significantly lower in the piscivore species (*H. malabaricus*) than in the other species ($P < 0.05$). There was no significant difference of the DSI and HSI among the species (table 1).

Table 1 - Parameters of the different fish species studied. IQ – intestinal quotient; HSI - Hepatosomatic index ; DSI - Digestive somatic index. Means + 1 SD.

Species	IQ	HSI	DSI
Hoplosternum littorale	1.34 ± 0.07a	1.23 ± 0.39a	2.02 ± 0.66a
Rhamdia quelen	0.73 ± 0.17b	1.69 ± 1.07a	3.94 ± 1.77a
Hoplias malabaricus	0.50 ± 0.06c	1.19 ± 0.39a	2.78 ± 0.64a

Means identified by different letters in the columns were significantly different ($P < 0.05$) as determined by ANOVA and Tukey test.

Plasma Cl⁻ levels were not significantly different among species. However, plasma Cl⁻ levels were higher than Cl⁻ levels in the fluid phase of the stomach content in H. malabaricus and than Cl⁻ levels in the fluid phase of all studied portions of digestive tract in H. littorale ($P < 0.05$). There was no significant difference on Cl⁻ levels among plasma and intestinal fluid phase of R. quelen. Chloride levels in the fluid phase of the stomach content of H. littorale were higher than in H. malabaricus ($P < 0.05$). The fluid phase of the anterior intestine content was higher in R. quelen than in the other species ($P < 0.05$), but there was no significant difference on Cl⁻ levels in the fluid phase of medium and posterior intestine contents of the studied species (table 2).

Table 2 – Chloride levels (mEq/L) in different compartments of the studied species. Means + 1 SD.

	H. littorale	R. quelen	H. malabaricus
Plasma	100.61 ± 10.77a	90.40 ± 16.25a	91.21 ± 13.57a
Stomach	66.94 ± 25.77a*	-	31.11 ± 20.76b*
Pyloric ceca or anterior intestine	47.76 ± 8.99a*	82.35 ± 16.64b	43.26 ± 13.17a
Medium intestine	-	52.02 ± 21.00a	72.50 ± 23.95a
Posterior intestine	26.89 ± 6.05a*	59.03 ± 40.89a	82.62 ± 63.87a

* significantly different from plasma in the same species ($P < 0.05$) as determined by one-way ANOVA and Tukey test

Means identified by different letters in the rows were significantly different ($P < 0.05$) as determined by ANOVA and Tukey test.

Conclusion

The obtained results showed that the IQ (but not DSI and HSI) can be an useful parameter to define the feeding habit. Moreover, Cl⁻ levels in the gastrointestinal tract are affected by feeding habit, and these levels can be different from plasma, indicating that ionic levels of the gastrointestinal content must be considered on studies of intestinal absorption.

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**CYTOCHEMICAL AND BIOCHEMICAL DETECTION OF
GLYCOCONJUGATES IN MUCOUS CELLS OF EURIHALINE FISH
(*Poecilia vivipara*) GILLS SUBMITTED TO SALINITY CHANGES**

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Abstract

It was used a panel of 12 lectins to evaluate the gills mucous cells behavior of euryhaline fish under stress condition of variations of salinity concentration (0 - 20ppm and 20 - 0ppm). The lectins distinguished 4 subtypes of mucous cells according to their glycoconjugate contents, which showed qualitative and quantitative changes either under increasing or decreasing the salinity concentrations. The quick response of mucous cells to turn-on and off their set of glycoconjugate secretion should be one of the mechanism related to adaptive capability of euryhaline fish and lectin cytochemistry showed to be a useful method to monitor them.

Background

The gill of teleost is a complex organ covered by an epithelia constantly exposed to the water containing microorganisms and several hazardous chemical compounds that potentially could affect their integrity. The mucous layer covering the gill epithelia seems to act as very sensitive biological cushion to adsorb or capture selectively the information from the contents of water (Perry & Laurent,1993). Despite of these facts, no consistent knowledge is available

neither about the composition of glycoconjugates contents of the mucous nor the mucous cells types responsible for its production. Sabóia-Morais et al., (1996) described 4 different mucous cells in the gill epithelia of the guppy, specie of euryhaline fish and suggested possible role related to the adaptive capability of these species in the environment salinity variations. The present work proposed to evaluate the plasticity of gill mucous cells responses against variations of salinity concentration in the euryhaline fish by carbohydrate cytochemistry.

Material and Methods

Adult male specimens of the guppy (*Poecilia vivipara*) were collected from seawater (SW) of Brazilian estuary (07° 42'S; 034° 50'W) with 20 ‰ salinity (SW₂₀) and from fresh-water (FW₀₀) of river without salinity traces measured by refractometer. The fishes captured from seawater (SW₂₀) were transferred in the proper tank containing seawater diluted with tap water at 10, 5 and 0 ppm of salinity. The fishes from fresh-water river (FW₀₀) were transferred into the tanks containing fresh water with increasing concentrations 5, 10 and 20ppm of salinity by adding a commercial salt complement used for seawater aquarium. After at least 6 h of adaptation period of salinity changes the gills from each experimental group were dissected and processed for conventional paraffin embedding, Spurr-resin embedding and tissue homogenates. The lectin histochemistry was performed with 12 biotinylated lectins (table 1) on paraffin sections. The Spurr-resin embedded ultra-thin sections collected on gold grids were submitted to periodic acid- thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining and analyzed under electron microscope. Tissue homogenates were used for SDS-PAGE and blotting for lectin reactions.

Results and Discussion

The 4 mucous cells subtypes showed affinity differences with lectins used and they were easily identified in the gill epithelia (table 2). These cells also showed qualitative and quantitative changes after submitted to the variations in the salinity concentrations. Subtype I cells showed constant positive reaction for UEA, but the intensity of reaction increased from SW₂₀ to SW₀₀ and decreased from FW₀₀ to FW₂₀. The subtype II cells localized at basal portion of gill lamellae, were reactive with most of the lectins used and showed large plasticity of responses under salinity variations. The subtype III mucous cells were the most common mucous cells of the guppy gills epithelia and showed reactivity

against almost all lectins used in this experiment, but the intensity of reaction in the mucous cells contents for each lectins, strongly varied between specimens submitted to salinity variation, both for FW and SW fishes. The subtype IV mucous cells were the only localized in the branchial rays and were positive for PHA-E₄ and LCA lectins in native FW₀₀ fishes, but the not in the native SW₂₀. The PA-TCH-SP reaction product was located in the small vesicles

Table 1 - Lectins and respective inhibitory sugars

Lectin	Source	Inhibitory sugars
LTA	Lotus tetragonolobus	L- α -Fucose
UEA	Ulex europaeus	L- α -Fucose
PNA	Arachis hypogea	D-GalNac
WGA	Triticum vulgare	D-GlcNAc
GSL-II	Griffonia simpliciflora	D-Gal-Nac
RCA-I	Ricinus communis	D-Lactose
RCA-II	<i>Ricinus communis</i>	β -Gal
VVA	Vicia vilosa	D-Gal-Nac
LCA	<i>Lens culinaris</i>	D-Man
PHA-E ₄	<i>Phaseolus vulgaris</i>	D-Gal-Nac
MAM	<i>Maackia amurensis</i>	Sialic acid
Con-A	<i>Canavalia ensiformes</i>	D-Man

of squamous cells, granules of subtype III mucous cells and through the luminal surface of gill epithelia of both SW and FW groups, but the reaction intensities were stronger in SW₂₀ than in FW₀₀ gills. The lectin panel cytochemistry showed to be a useful tool to demonstrate the wide diversity in the gill's mucous cells secretion. This mucous secretions were deeply affected by environmental conditions, as were demonstrated the different responses of fishes submitted to experimental salinity variations. Unfortunately, these differences were not reproducible in the gill homogenates fractioned by SDS-PAGE and reacted with same lectins. It probably means the lectin reactive glycoconjugates detected in the cytochemistry, could be glycosaminoglycan like molecules rather than glycoproteins. The heterogeneity in the glycoconjugate contents of subtypes II and III cells elect them as most sensitive and higher plasticity of responses against salinity variations. Therefore, the

capability of the fishes to adapt in the salinity variation (Nolan et al, 1999) could be due to the inherited ability of the gills mucous cells much more developed in the euryhaline fish.

(FW₀₀₋₂₀) -Fresh-water fishes adapted from 0 to 20 ppm salinity and SW₂₀₋₀₀ Seawater fishes adapted from 20 to 00 ppm salinity. (+++) strong. (++) moderate; (+) weak and (-) negative reactions

Acknowledgements

This work was partially supported by FAPESP, CNPq, FUNAP-UFG and CAPES.

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Table 2- Lectin reactivity of gill mucous cells of *Poecilia vivipara* under salinity variations

M	0/00	LT	UE	PN	WG	GSL	R	VV	LCA	PH	MA	Com	R
C		A	A	A	A	II	C	A		A-E4	M	A	C
							A						A
							I*						II
													*
	FW ₀₀	-	++	-	-	-	-	-	*	-	-	-	-
	FW ₀₅	-	++	-	-	-	-	-	-	-	-	-	-
I	FW ₁₀	-	+	-	-	-	-	-	-	-	-	+/-	-
	FW ₂₀	++	+	-	-	-	-	-	-	-	-	-	-
	FW ₀₀	++	-	-	-	-	-	-	++	+	-	+++	-
	FW ₀₅	++	-	+	-	+	-	-	++	+	++	+++	-
II	FW ₁₀	++	+	++	-	+	-	-	++	+	-	+++	-
	FW ₂₀	+++	+	++	-	++	-	+	++	+++	-	+++	-
	FW ₀₀	+	-	-	+	-	-	-	-	+	-	-	-
	FW ₀₅	+	-	+	+	+	-	+	+	++	-	-	-
III	FW ₁₀	++	-	+	-	-	-	-	+	++	-	-	-
	FW ₂₀	++	+	+	-	++	-	++	++	++	++	-	-
	FW ₀₀	-	-	-	-	-	-	-	+	+	-	-	-
	FW ₀₅	-	-	-	-	++	-	-	++	+	-	-	-
IV	FW ₁₀	-	-	-	++	-	-	-	++	-	-	-	-
	FW ₂₀	+++	+	+++	-	-	-	-	-	-	+	+++	-
	SW ₂₀	-	+	-	-	-	-	-	-	-	-	-	-
	SW ₁₀	+	+	-	-	-	-	-	-	-	-	-	-
I	SW ₀₅	+	++	-	-	-	-	-	-	-	-	-	-
	SW ₀₀	+	+++	-	-	-	-	-	-	-	-	-	-
	SW ₂₀	+	-	+	-	+++	-	++	*	++	-	-	-
	SW ₁₀	+	-	+	-	++	-	++	-	++	-	-	-
II	SW ₀₅	+	+	++	-	++	-	++	-	++	++	-	-
	SW ₀₀	+	++	+++	-	+++	-	++	*	++	-	-	-
	SW ₂₀	-	-	+	+	+	-	+	-	++	-	-	-
	SW ₁₀	+	-	+	-	+	-	+	-	+	-	-	-
III	SW ₀₅	+	-	+	-	++	-	+	-	+	-	-	-
	SW ₀₀	+	-	++	-	++	-	+	*	+	-	-	-
	SW ₂₀	-	-	-	-	-	-	-	-	+	-	-	-
	SW ₁₀	-	-	+	-	-	-	+	-	+	+	-	-
IV	SW ₀₅	-	-	-	-	+++	-	++	-	+	-	-	-
	SW ₀₀	-	-	-	-	-	-	-	-	+	-	-	-

**MODULATION OF BRANCHIAL ION TRANSPORT PROTEIN
EXPRESSION BY SALINITY IN THE
EURYHALINE GREEN PUFFERFISH (*TETRAODON FLUVIATILIS*)**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

The green puffer fish (*Tetraodon fluviatilis* Hamilton 1822) is a brackish water teleost native to estuaries and the fresh water tidal zone of rivers in South East Asia from India to Indonesia (Rainboth 1996). Like other pufferfishes (Fugu, Tetraodon), the genome of *T. fluviatilis* is small in size. This coupled to the fact that it is readily available through the aquarium fish trade has made it the subject of interest for genomic studies (e.g. Cnogorac-Jurcevic et al. 1997). However, despite this recent surge in interest other aspects of its biology remain poorly studied. The description of the salinity tolerance of this species in the aquarium fish literature is at best confusing due to its resemblance to the freshwater puffer (*T. nigroviridis*).

In the present study, we investigated the salinity tolerance of *T. fluviatilis* and some ionic regulatory mechanism that might be modulated by salinity.

Materials and Methods

Juvenile pufferfish were purchased from an aquarium shop in TungChoi Street, Kowloon HongKong. In the laboratory they were acclimated to freshwater

(FW), 5, 15, 30 and 48 ‰ salinity for 10 days. Na⁺/K⁺-ATPase and V-ATPase activities were measured through the use of the specific inhibitors ouabain and bafilomycin A1, respectively, in an in vitro assay system. In addition, Na⁺/K⁺-ATPase protein level expression was measured using immunoblotting and cell specific expression using immunofluorescence (IF) microscopy. Cell specific expression of CFTR and Na⁺:K⁺:2Cl⁻ cotransporter (NKCC) were also investigated using IF. (see Wilson et al. 2004 for details)

Results and Discussion

T. fluviatilis acclimated to the different salinities without mortality, however in preliminary experiments FW acclimated fish tended to develop parasitic infections. Branchial Na⁺/K⁺-ATPase activity was significantly elevated only in hypersaline SW (Fig1). However, Na⁺/K⁺-ATPase protein level expression was significantly elevated at 32‰ SW as well.

Branchial V-ATPase activity was not affected by salinity. This would indicate that the V-ATPase does not have an important role in FW ion uptake.

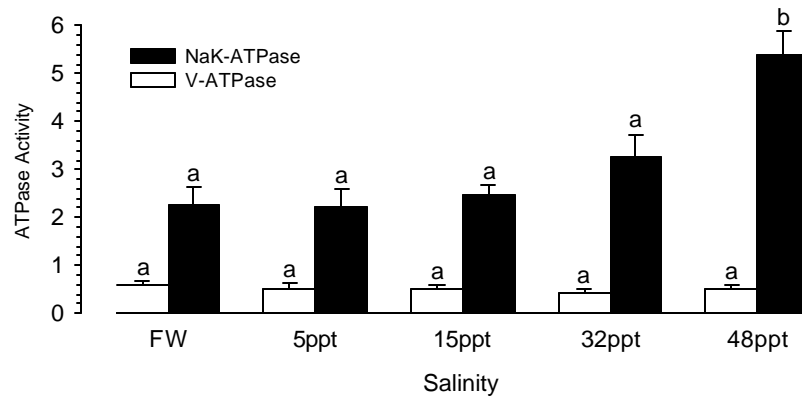


Figure 1. Na⁺/K⁺-ATPase and V-type H⁺-ATPase activities in crude gill homogenates of *Tetraodon* acclimated to different salinities. Bars with like characters are not significantly different

Branchial NKCC protein level expression was significantly elevated at high salinity. Na^+/K^+ -ATPase and NKCC immunoreactive (IR) cells increased in size and intensity of IF with salinity. These cells were also more rounded in FW. The number of Na^+/K^+ -ATPase IR cells increased in the interlamellar region but not in the flat trailing edge of the filament. Apical CFTR expression (number of cells and intensity of IF) increased in a salinity dependent manner (Fig2).

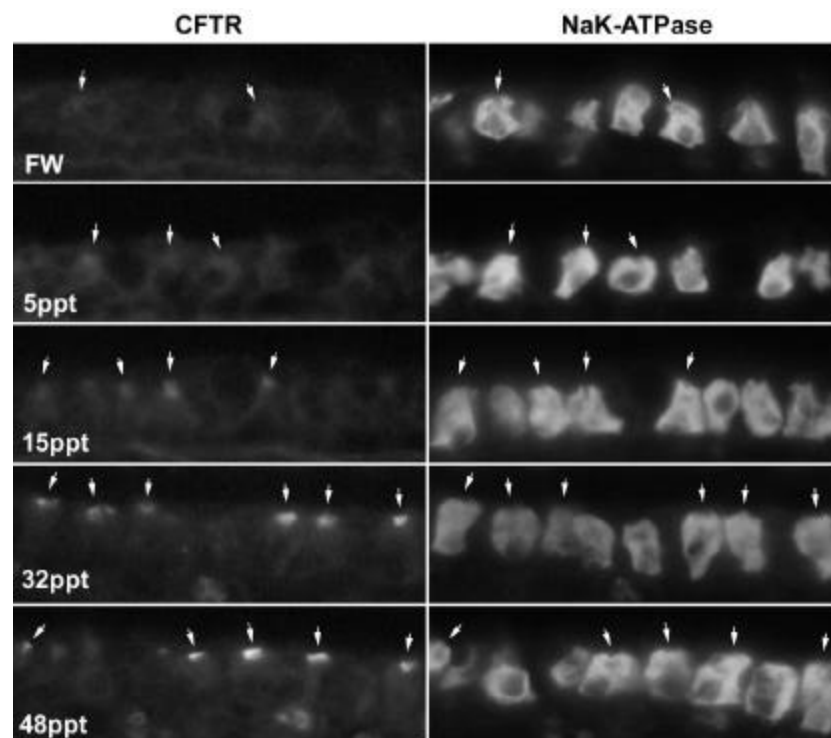


Figure 2. Double immunofluorescence labeling of *T. fluviatilis* gill filaments from animals acclimated to different salinities. Arrows indicate cells with apical CFTR and basolateral Na^+/K^+ -ATPase immunoreactivity.

The response of Na^+/K^+ -ATPase, NKCC and CFTR to salinity is similar to that reported in other species. However, neither Na^+/K^+ -ATPase nor V-ATPase

activity were increased following acclimation to FW. This latter finding might partial explain why this species is reported not to fare very well in freshwater when kept in captivity.

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ONTOGENETIC AMMONIA EXCRETION
IN SILVER CATFISH *Rhamdia quelen* (HEPTATERIDAE)

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The main nitrogenous residue eliminated by fish is ammonia, which is toxic at low concentrations. Therefore, even at the beginning of their life stage fish must be able to excrete excess nitrogen. The objective of the present study was to verify ammonia excretion in different life stages (eggs, larvae, and juveniles) of silver catfish (*Rhamdia quelen*). In addition, the influence of fasting on ammonia excretion was also investigated.

Methodology

Silver catfish eggs (mean weight 5.0 mg) were obtained from induced spawning at the fish culture sector of the Universidade Federal de Santa Maria. Thirty eggs were placed in continuously aerated 20 mL chambers (ten replicates), and 12, 24, 48, and 198 h after fecundation water from these chambers was collected and stored at -20°C for posterior analysis of ammonia concentration. Eggs and larvae were weighted after each water collection. Larvae received food every two hours after hatching (around 28 h after fecundation). Silver catfish juveniles were separated in three classes of weight: 2 – 50 g, 50 – 150 g, and 150 – 320 g. After feeding, fish were placed in individual chambers with approximately 20

times their volume in water. Water was collected from each chamber at time 0, 6, 12, 24, 36, and 48 h after transference, and stored at -20oC for posterior analysis. Fish were not fed when inside the chambers. Ammonia concentration in the water samples were measured according to Verdouw et al. (1978). Comparisons among ammonia excretion at different times of fasting were made by one-way ANOVA and Tukey test with the aid of the software Statistica 5.0. Correlation between silver catfish weight and ammonia excretion was performed with the software Slide Write Plus 4.0.

Results

Ammonia excretion by the eggs was low, but when hatching started it increased up to 48 h after fecundation. Larger larvae presented lower ammonia excretion (figure 1).

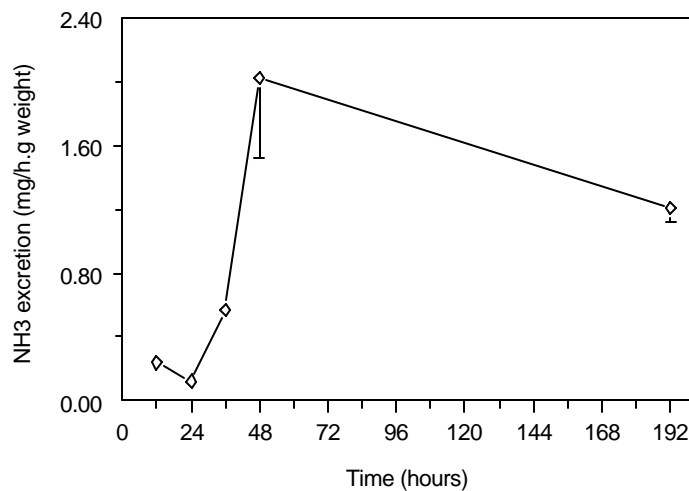


Figure 1 – Ammonia excretion in silver catfish eggs and larvae.

There was a significant correlation between ammonia excretion after 48 h of fasting and weight: ammonia excretion per unit of body mass was higher in the small specimens than in the larger ones. This correlation is given by the following equation:

$$y = -0.976 + 2.163/(1 + (x/461.57)^{0.42}) \quad r^2 = 0.960$$

where y = ammonia excretion ($\mu\text{g/h}$. g weight) and x = fish weight (g)

Ammonia excretion was significantly higher after 6 h of fasting than after 12 h in the smaller specimens, and significantly higher after 6 h than after 48 h of fasting in the largest specimens ($P < 0.05$) (figure 2).

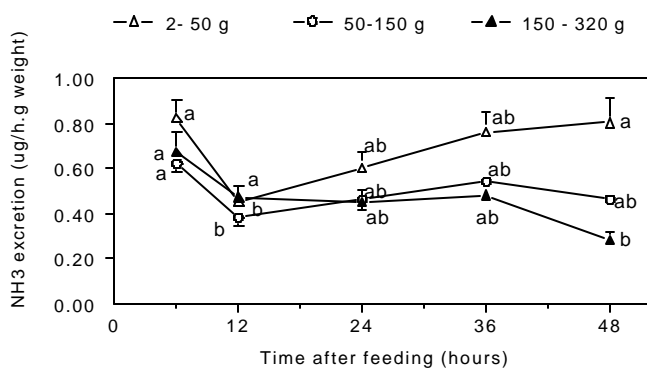


Figure 2 – Ammonia excretion in silver catfish as a function of time of fasting.

Conclusion

The higher ammonia excretion at hatching time (28-48 h) probably occurred due to the higher elimination of metabolites during this process. The higher ammonia excretion per unit of body mass in smaller than in larger specimens of silver catfish is in agreement with the fact that metabolic rate decreases as body size increases. Ammonia excretion can change in the first 12 h of fasting in

smaller specimens but only after 48 h in larger ones, which would have a lower metabolic rate.

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**ROLE OF THE BRANCHIAL NKCC
IN THE TRANSITION BETWEEN
HYPO- AND HYPER-REGULATION
IN THE ESTUARINE PUFFERFISHES
Sphoeroides testudineus AND *S. greeleyi***

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter (NKCC) is present in most cell types and epithelia, performing either secretion (NKCC1) or absorption (NKCC2) of NaCl. Loop diuretics such as furosemide inhibit the activity of both isoforms. The pufferfish *Sphoeroides testudineus* Linnaeus, 1758 (St) is an euryhaline species in the estuaries of Brazil, found in waters where salinity ranges from 34 (tidal plains) to 0 (tidal creeks). *S. greeleyi* Gilbert, 1900 (Sg), is also found along the coast of Brazil in shallow bays and estuaries and has been observed to be restricted to areas of higher salinity than *S. testudineus*. Both species are excellent plasma ion regulators, in a range from 35-5 salinity, even after 5 days of exposure. These fishes are daily submitted to a wide variation in salinity due to the tidal cycle, switching from hypo- to hyper-regulation within a time span of ~6 hours. Mechanisms through which the transition between hypo- and hyper-regulation takes place in estuarine teleosts are not well known, and the investigation of the localization/ expression of branchial transport systems may contribute to the building of a model for that transition.

The objective of this study was to investigate the effect of furosemide injection on hypo-regulation (when in 35 seawater) and hyper-regulation (in 5 seawater) and the effect of salinity on the abundance of NKCC in the gill epithelium, as detected by immunocytochemistry.

Materials and Methods

Fish were acclimated for either 6h or 5 days (5d) in 35 or 5 seawater (SW). After these periods, 1 mL of either furosemide (10 mg/mL) (furosemide injection-FI) or a 300 mOsm.kgH₂O⁻¹ NaCl saline (Saline injected -SI) was injected (intra-muscle). One hour post injection, blood samples were obtained for determination of plasma osmolality ([Osm]), chloride ([Cl⁻]), and hematocrit (Hct), through standard methods. Muscle samples were obtained for determination of muscle water content (H₂O%). One left gill arch was removed and processed according to standard protocols of immunocytochemistry. The monoclonal primary antibody T4 for NKCC (DSHB, Iowa) and a secondary antibody (fluorescein-labeled) goat anti-mouse (Pierce[®]) were employed. Statistical analysis of the data was done using the software SigmaStat[®] version 2.03, always with significance level set at 0.05. “TWO-WAY ANOVA” were performed to evaluate osmolality, chloride, hematocrit and water muscle content, followed by the *post hoc* test of Tukey. Mann-Whitney tests were employed for the analysis of fluorescence intensity, always within a species.

Results and Discussion

FI lead to increased [Osm] and [Cl⁻] in 35 SW and to decreased values in 5 SW, for both species, after 6h and 5d. These results are compatible with NKCC inhibition during hypo-regulation (in 35 SW), as would be expected, and a possible inhibition of NKCC during hyper-regulation (in 5 SW). FI lead to no significant effects on Hct and muscle H₂O%, probably meaning lack of effect on volume regulation of either blood cells or muscle tissue (Table 1).

Immunocytochemistry revealed the presence of NKCC in the inter-lamellar region of the gill epithelium of St and Sg acclimated to 35 SW and 5 SW, for both 6h and 5d. However, there was a significant reduction in fluorescence intensity in St after 5d in 5 SW, when compared to the signal in 35 SW (Figure 1).

Table 1: Furosemide injected (F) and Saline injected (S) *S. testudineus* and *S. greeleyi* maintained for 6h or 5d in 5 or 35 SW. Different letters in the superscript indicate statistical differences, within the same species.

		[Osm] mOsm/kg.H ₂ O	[Cl] mM	Hct %	H ₂ O %
S. testudineus	S 5sw/6h	316.0±10.3 ^a	148.1±7.9 ^b	29.5±2.0	29.5±2.0 _a
	F 5sw/6h	295.5±8.3 ^a	118.4±4.8 ^a	22.7±1.5	22.7±1.5 _b
	S 35sw/6h	337.4±4.5 ^a	163.2±3.6 ^b	25.2±2.0	25.2±2.0 _a
	F 35sw/6h	388.7±14.5 ^b	173.1±8.8 ^b	26.3±2.3	26.3±2.3 _a
	S 5sw/5d	314.5±3.3 ^a	145.5±5.2 ^b	31.7±0.8	31.7±0.8 _b
	F 5sw/5d	288.5±9.3 ^a	117.3±7.5 ^a	24.7±1.3	24.7±1.4 _c
	S 35sw/5d	321.5±5.5 ^a	166.2±5.7 ^b	31.0±0.8	31.0±0.8 _b
	F 35sw/5d	394.7±14.5 ^b	169.2±10.1 ^b	18.7±1.8	18.7±1.8 _a
S. greeleyi	S 5sw/6h	319.8±4.9 ^b	155.5±11.1 ^b	24.0±1.6	24.0±1.6
	F 5sw/6h	275.3±7.3 ^a	126.1±5.4 ^a	21.5±1.6	21.5±1.6
	S 35sw/6h	358.1±18.3 ^b	192.0±9.8 ^c	20.7±1.9	20.7±1.9
	F 35sw/6h	425.0±21.1 ^c	187.1±10.0 ^c	21.0±3.0	21.0±3.0
	S 5sw/5d	328.8±14.4 ^b	152.1±7.8 ^b	20.2±0.9	20.2±0.9 _b
	F 5sw/5d	289.3±3.4 ^a	130.2±6.8 ^a	19.8±1.2	19.8±1.2 _a
	S 35sw/5d	360.7±10.5 ^b	178.3±9.6 ^c	21.6±1.2	21.6±1.2 _b
	F 35sw/5d	414.2±10.4 ^c	179.0±5.2 ^c	22.3±1.1	22.3±1.1 _a

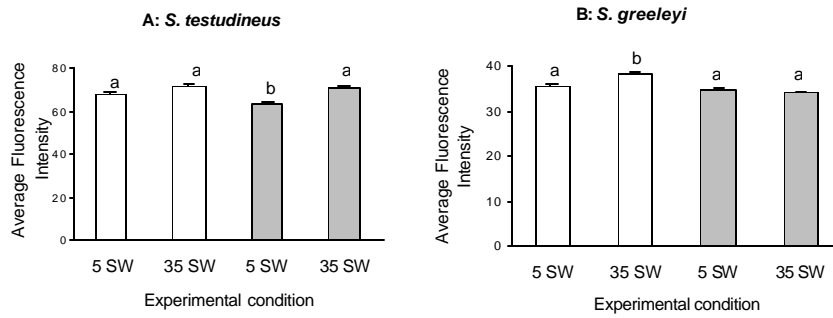


Figure 1: Average fluorescence intensity of NKCC in *S. testudineus* (4<n<6) (A), and *S. greeleyi* (3<n<5) (B). Different letters over the bars indicate significant differences. White bars: 6h; Filled bars: 5d.

**WATER pH AND URINARY EXCRETION
IN SILVER CATFISH (*Rhamdia quelen*)**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Urinary bladder and kidney play an important role on freshwater teleost osmoregulation because the kidney eliminates the excess of water that enters the fish by osmosis, and the urinary bladder reabsorbs ions. In addition, it is known that water pH can change the ionic equilibrium of teleosts, but the role of kidney and urinary bladder in this process has not been studied yet. Therefore, the aim of the present study was to analyze the effect of the transference to different water pH on the urinary excretion of silver catfish (*Rhamdia quelen*).

Methodology

Silver catfish specimens (20-30 cm) were previously adapted for one week in continuously aerated 250 L freshwater tanks (22°C, pH 7.5, water hardness 30 mg/L CaCO₃). Fish were anaesthetized with clove oil (32 mg/L) and the urinary bladder cannulated. After, fish were transferred to a 2 L chamber with continuous water flow furnished by an immersion pump. Fish were exposed to pH 7.5, 4.0, 5.0, 8.0, or 9.0. Water pH was lowered with 10% sulfuric acid and increased with 10% NaOH. Urine was collected throughout four days, and within this period fish were fasting. Urine pH was measured with a Hanna pHmeter and after samples were frozen at -20°C for posterior analysis of Cl⁻ and ammonia concentrations. Chloride concentration was measured according to Zall et al. (1956) and ammonia following the technique of Verdouw et al.

(1978). Correlations between water pH and urine pH, Cl⁻ or ammonia concentration were made with the software Bioestat 2.0.

Results

There was a significant decrease on the urinary excretion of ammonia and Cl⁻ with the increase of silver catfish weight. There was also a significant decrease on urinary ammonia excretion with increase of water pH (figure 1), but there was no significant correlation between water pH and urinary Cl⁻ concentration. Correlation between amount of excreted Cl⁻ by urine volume was significant in all studied water pHs (increase of urinary Cl⁻ excretion with increase of urine volume), but amount of excreted ammonia by urine volume showed a good correlation only at water pH 4.0, 5.0 and 7.5 (r² around 0.8). The increase of water pH also increased significantly urine pH (figure 2).

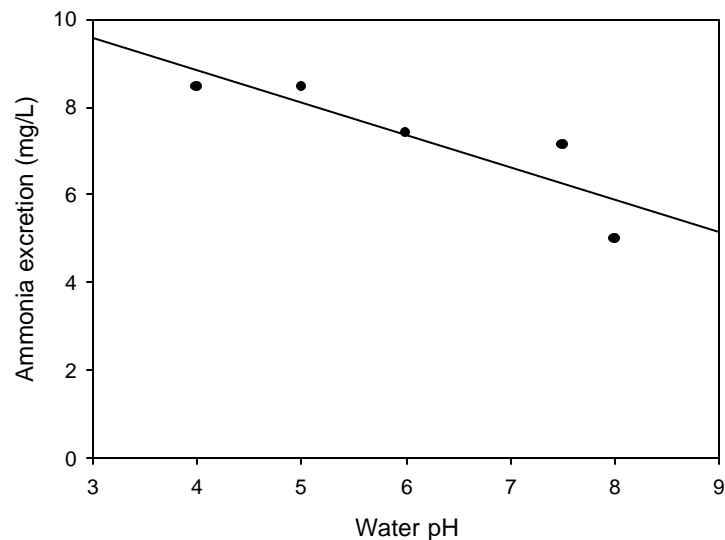


Figure 1 – Urinary ammonia excretion as a function of water pH. The following equation was fitted to the data: $y = 11.80 - 0.740x$ $r^2 = 0.771$ Where y = urinary ammonia excretion (mg/L) and x = water pH

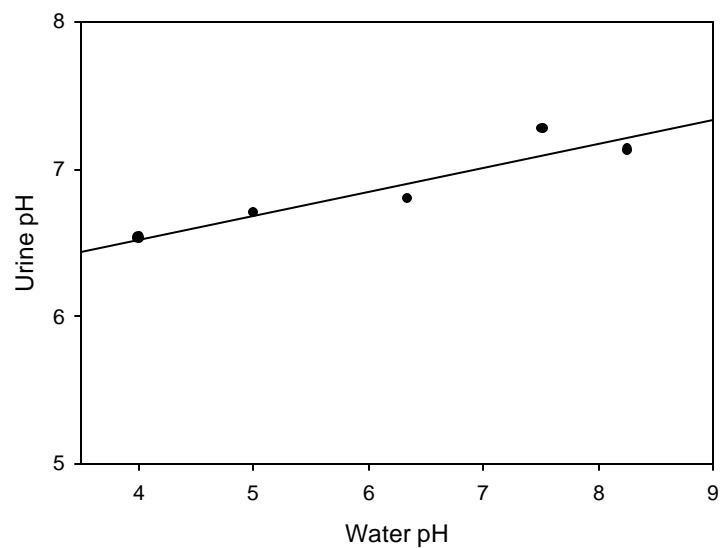


Figure 2 - Urine pH as a function of water pH (urine from fish exposed for 32-48 h to the experimental pH). The following equation was fitted to the data: $y = 5.87 - 0.163x$ $r^2 = 0.870$ Where y = urine pH and x = water pH

Conclusion

The decrease of Cl^- and ammonia urinary excretion with increase of silver catfish weight might be related to the fact that larger fish present lower metabolism, and therefore urinary excretion decreases. It can also be concluded that urinary excretion in silver catfish is changed by water pH, indicating that kidney and urinary bladder could participate on the adaptation of silver catfish to acidic or alkaline environments.

Acknowledgements

K.C.S. Bolner was the recipient of a CNPq fellowship.

References

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