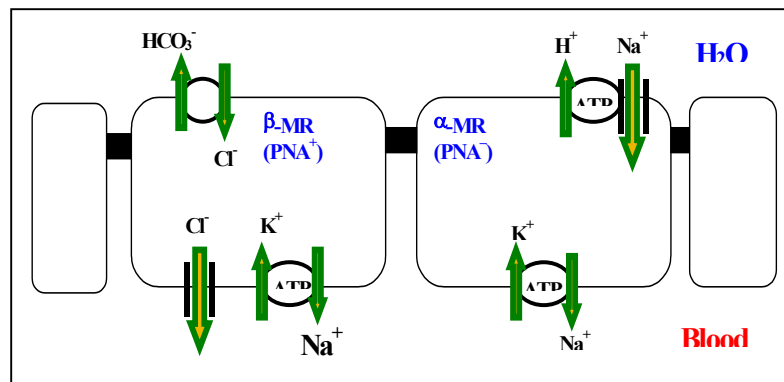


# ***Ion Regulation***

## ***In Fish***



**Steve McCormick**

**Don M<sup>ac</sup>Kinlay**

*International Congress on the Biology of Fish*  
*University of British Columbia, Vancouver, CANADA*



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SYMPOSIUM PROCEEDINGS

**Steve McCormick**

**Don M<sup>ac</sup>Kinlay**

*International Congress on the Biology of Fish*  
*University of British Columbia, Vancouver, CANADA*

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International Standard Book Number (ISBN) 1-894337-34-4

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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. Although we have a basic understanding of some of the mechanisms involved in ion regulation, recent studies indicate that there is significant diversity among teleosts in the transporters involved in ion regulation, their localization and control. This symposium focused on recent advances in the osmoregulatory physiology of fishes.

Symposium Organizers:

Steve McCormick, Conte Anadromous Fish Laboratory, Turners Falls MA

Don MacKinlay, Fisheries & Oceans Canada, Vancouver

## CONGRESS ACKNOWLEDGEMENTS

This Symposium is part of the International Congress on the Biology of Fish, held at the University of British Columbia in Vancouver B.C., Canada on July 22-25, 2002.

The sponsors included:

- Fisheries and Oceans Canada (DFO)
- US Department of Agriculture
- US Geological Service
- University of British Columbia Fisheries Centre
- National Research Council Institute for Marine Biosciences
- Vancouver Aquarium Marine Science Centre

The main organizers of the Congress, on behalf of the Physiology Section of the American Fisheries Society, were Don MacKinlay of DFO (overall chair, local arrangements, program and proceedings) and Rosemary Pura of UBC Conferences and Accommodation (facility arrangements, registration and housing). Thanks to Karin Howard for assistance with Proceedings editing and word-processing; to Anne Martin for assistance with the web pages; and to Cammi MacKinlay for assistance with social events.

I would like to extend a sincere 'thank you' to the many organizers and contributors who took the time to prepare a written submission for these proceedings. Your efforts are very much appreciated.

Don MacKinlay  
Congress Chair

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**OSMOREGULATION IN AMERICAN SHAD; READJUSTING  
EXPECTATIONS OF MIGRATORY PHYSIOLOGY**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

The study of migratory fish physiology has a rich history that has often focused on the development of salmonids through their downstream migratory period. The parr-smolt transformation has been well characterized and its study stands as a most impressive example of the interface of behavioral, physiological and ecological approaches to biology. For salmonids, the development of seawater tolerance, changes in morphology and migratory behavior are largely coordinated. As such, there is a temptation to transpose these patterns on other species. The purpose of this work is to explore the migratory physiology of American shad, *Alosa sapidissima*. This prominent anadromous fish deviates widely from the salmonid model.

Adult shad spawn in fresh water in the spring and young shad develop the ability to enter into full strength seawater at the larval-juvenile transition, months prior to downstream migration (Zydlewski and McCormick, 1997a). This development is coincident with gill development, increased gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and differentiation of gill chloride cells (Zydlewski and McCormick, 2001). While juvenile shad are physiologically competent to enter seawater in the summer of their fresh water residence, most remain in fresh water until autumn.

While shad maintain seawater tolerance through the autumnal period of seaward migration they gradually lose the ability to regulate their ions in fresh water. This is evidenced by decreased plasma chloride, decreased plasma osmolality, increased muscle moisture and high mortality if prevented from entering

seawater (Zydlewski and McCormick, 1997b; unpublished data). Associated with impaired hyperosmoregulatory ability is an increase in gill  $\text{Na}^+, \text{K}^+$ -ATPase activity which is the result of an intense proliferation and enlargement of  $\text{Na}^+, \text{K}^+$ -ATPase-rich chloride cells on both the primary filament and secondary lamellae of the gill (Zydlewski and McCormick, 2001). The disappearance of chloride cells on the secondary lamellae upon seawater entry indicates that the function of these cells is likely to be in ion uptake. The nature of this developmental loss of hyperosmoregulatory ability is not understood, but the clear parallel acceleration of physiological changes under declining temperature through autumn implies a direct relationship between osmoregulatory ability in fresh water and downstream migratory behavior.

In nature, migration is protracted over several months and over a considerable temperature range. There is no apparent physiological disadvantage to migrating early while river temperatures are warm but the impending osmoregulatory changes associated with declining temperature in fresh water may impact the migratory success of late migrants. In captivity, a cessation of feeding below 10 °C in juvenile shad has been observed and other sub-lethal and lethal effects occur at lower temperatures in fresh water. During the period of migration, juvenile shad also exhibit a heightened responsiveness to acute handling and confinement stress (Shrimpton et al, 2001). These effects are likely due to osmotic stress.

These behavioral and physiological effects are likely to impair downstream migratory success through osmotic perturbation upon seawater entry. Indeed, juveniles iso-thermally transferred to 24 ppt seawater in September (24°C; "early") and in November (10°C; "late") demonstrated markedly different abilities to acclimate to seawater. Early acclimation resulted in a modest osmotic perturbation that recovered rapidly. Hematocrit declined 14% at 24h, recovering within 48 h. Plasma osmolality increased 6% at 4h, recovering within 8 h. Early acclimation caused a 2-fold increase in gill  $\text{Na}^+, \text{K}^+$ -ATPase activity by 24 h and a 4-fold increase by 4 d. The number of chloride cells on the primary gill filament increased 2-fold by 4 d. Chloride cells on the secondary lamellae rapidly decreased from 22 cells/mm to less than 2 cells/mm within 4 d. Late acclimation resulted in a severe and protracted osmotic perturbation. Hematocrit levels declined 23% by 4 d, recovering by 14 d. Plasma osmolality increased 36 % by 48 h, recovering by 4 d. Initial gill  $\text{Na}^+, \text{K}^+$ -ATPase activity was 2-fold greater than in early fish and did not change during acclimation. Initial number of chloride cells on the primary filament was 2-fold greater than early fish and did not increase during acclimation. Initial

number of chloride cells on the secondary lamellae was 5-fold greater than early fish (116 vs 22 cells/mm) declining to negligible numbers over 14 d.

These data indicate a unique pattern of behavior and physiology in juvenile American shad. Not only does the physiological ability to enter into seawater greatly precede migration, but migrant fish exhibit impaired osmoregulatory ability in fresh water. In fact, late migrants face an even greater physiological challenge during seawater acclimation. Physiological performance, together with ecological factors (such as predation and food availability) apparently stabilizes the timing of autumnal migration.

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**ASIAN MEDAKAFISHES: IDEAL MODELS FOR ANALYSES  
OF SEAWATER ADAPTABILITY**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

The genus *Oryzias* is a group of small fishes that consists of more than 14 diverse species including Japanese medaka (*O. latipes*), a well-known laboratory animal (Roberts, 1998). This genus is expected to offer good models for studying mechanisms of osmotic adaptation because it contains freshwater (FW) and seawater (SW) species. In this study, we compared SW adaptability of four species, *O. javanicus*, *O. dancena*, *O. latipes* and *O. marmoratus* inhabiting different osmotic environments (Table 1). SW adaptability was evaluated using several indices including survival of adult fish after transfer from FW to SW and fertilization and hatching rates in SW (Inoue and Takei, 2002).

**Survival of adult fish after transfer from FW to SW**

In the first experiment, the survival rate of adult fish after direct transfer from FW to SW or 50%SW was compared. *O. javanicus* and *O. dancena* revealed high adaptability to sudden increase in the ambient osmotic pressure and survived the transfer to SW. In contrast, all *O. marmoratus* and *O. latipes* transferred to SW died within 2 hr and 9.5 hr, respectively. *O. marmoratus* was not adaptable even to 50% SW; all fish died within 25 hr after transfer. On the other hand, *O. latipes* survived in 50% SW. It was also found that prior acclimation of *O. latipes* in 50% SW for 24 hr allowed them to survive in SW. This result suggests that *O. latipes* is potentially euryhaline but only lacks the adaptability to a sudden increase of the ambient osmotic pressure. This characteristic is especially interesting for studying osmoregulatory mechanisms because it becomes possible, using this species, to study the functions of fast-acting osmoregulatory factors such as natriuretic peptides that may act immediately after encountering the environment with different salinity (Takei and Hirose, 2002).

Table 1. Adaptability to seawater in four *Oryzias* species

Species	<i>Oryzias marmoratus</i>	<i>Oryzias latipes</i>	<i>Oryzias dancena</i>	<i>Oryzias javanicus</i>
Geographic Distribution	Lake Towuti in Sulawesi Island	Japan, Korea and China	Pakistan–Thailand	Malaysian Peninsula–Indonesia
Habitat	FW	FW, BW	BW, FW	BW, SW
Immediate adaptation to SW	–	–	++	++
Slow adaptation to SW	–	++	++	++
Spawning in SW	N.D.	++	++	++
Fertilization in SW	N.D.	+	++	++
Sperm motility in SW	±	±	++	++
Embryonic development in SW	–	+	++	++
Hatching in SW	–	++	+	++

++, possible; +, possible in low rate; ±, hardly possible; –, impossible; N.D., no data.

### **Fertilization rates in FW and SW**

*O. latipes* spawned eggs everyday while other three species spawned several times a week in FW. More than 90% eggs of all four species were found to be fertilized. *O. latipes*, *O. dancena* and *O. javanicus* also spawned eggs in SW as frequently as in FW. Most eggs of *O. dancena* and *O. javanicus* spawned in SW were fertilized as in FW. However, 84% of *O. latipes* eggs spawned in SW remained unfertilized. By microscopic observation, we found that the sperm of *O. javanicus* and *O. dancena* is motile both in FW and SW. In contrast, the sperm of *O. latipes* and *O. marmoratus* is motile in FW but hardly motile in SW. It is well known that the sperms of SW fish are activated when they are exposed to water with osmotic pressure or concentration of ions such as  $K^+$  higher than the seminal plasma, while those of FW fish are activated by lower osmotic pressure or ion concentration (Billard and Cosson, 1992). The sperm of *O. dancena* and *O. javanicus*, which is motile both in FW and in SW, is quite unique and offers a new model to study sperm activation mechanisms.

### **Hatching rates in FW and SW**

The normally fertilized eggs of the four species spawned in FW were incubated in SW or FW and the embryonic development and hatching were monitored. The hatching rate in SW was the highest in *O. javanicus* (84%), followed by *O. latipes* and *O. dancena* (60%), and the lowest in *O. marmoratus* (0%). The low hatching rate of *O. marmoratus* and *O. latipes* embryos in SW was due to the death of embryos before reaching the hatching stage. In contrast, embryos of *O. dancena* developed successfully to hatching stage but failed to hatch out. One possible cause of the low hatching ability of *O. dancena* in SW is that hatching enzyme does not function in high salinity. Molecular cloning of cDNAs encoding hatching enzymes of *O. javanicus* and *O. dancena* is now in progress to compare enzyme activities under the different salt concentration.

### **Future studies**

*O. latipes* is known to have various advantages for experimental purposes, e.g., its small size, frequent spawning, and short generation time, etc. We found that other three species, share such advantages with *O. latipes*. Thus, it seems possible to apply to these species experimental approaches including foreign gene transfer (Ozato *et al.*, 1989). We expect that the application of such experimental techniques to various *Oryzias* species (e.g., overexpression of natriuretic peptide genes in *O. latipes*, transfer of *O. javanicus* hatching enzyme genes into *O. dancena*, etc.) would bring us new

information to understand how fish has developed diverse mechanisms to adapt to hyper- and hypo-osmotic environments.

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### **Acknowledgements**

We thank Drs. J. Koyama, A. Shima, A. Shimada, K. Naruse and M. Sakaizumi for kind gifts of fish strains and helpful advice. We are grateful to Drs. M. Nishida and S. Yasumasu for helpful discussion.

## CALCIUM BALANCE IN DEVELOPING FISH

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### EXTENDED ABSTRACT ONLY - DO NOT CITE

#### Introduction

Freshwater teleosts maintain their plasma  $\text{Ca}^{2+}$  levels within a narrow limit (2-4 mM) in a wide range of external  $\text{Ca}^{2+}$  concentrations. Gill mitochondria-rich (MR) cells (or chloride cells) are the major sites for  $\text{Ca}^{2+}$  uptake from freshwater environments, and active  $\text{Ca}^{2+}$  uptake in response to low ambient  $\text{Ca}^{2+}$  is therefore a prerequisite for  $\text{Ca}^{2+}$  homeostasis. Developing fish embryos or larvae, whose organ systems are under or poorly developed, have to face freshwater environments with a wide range of external  $\text{Ca}^{2+}$  levels as their adults do. The present work was aimed to study the mechanisms how fish embryos and larvae maintain the body  $\text{Ca}^{2+}$  balance in freshwater environments.

#### Materials and methods

Fertilized eggs or hatched larvae of tilapia (*Oreochromis mossambicus*) goldfish (*Carassius auratus*), zebrafish (*Danio rerio*) and ayu (*Plecoglossus altivelis*) were incubated in low- $\text{Ca}^{2+}$  (0.02 mM), mid- $\text{Ca}^{2+}$  (0.2 mM), and high- $\text{Ca}^{2+}$  (2 mM) for different periods of time depending on experimental designs. For long-term acclimations, fertilized eggs were incubated with different levels of  $\text{Ca}^{2+}$ , and for the acute exposure experiments larvae with different ages were transferred from mid- $\text{Ca}^{2+}$  to low- $\text{Ca}^{2+}$  or low- $\text{Ca}^{2+}$  freshwater. Animals were sampled for the measurements of  $\text{Ca}^{2+}$  fluxes and  $\text{Ca}^{2+}$  contents by radio  $^{45}\text{Ca}^{2+}$  tracing and atomic spectrometry, respectively.

### **Ontogeny of Ca<sup>2+</sup> balance**

Tissue Ca<sup>2+</sup> content in tilapia remained at a constant level during the embryonic stages and showed dramatic changes after hatching; tissue Ca<sup>2+</sup> increased about 30-35 fold during 10 d after hatching. Both Ca<sup>2+</sup> influx and efflux in whole body of tilapia significantly increased following larval development. However, the extent of increase was much higher in influx (4.3 fold) than in efflux (2.3 fold), resulting in a 4.6-fold increase of Ca<sup>2+</sup> net uptake rate within 1 week after hatching. A continuous increase in body calcium content with larval development may be critical for the normal growth and development of larvae.

### **Acclimation to low-Ca<sup>2+</sup> environment**

Upon acclimation to low-Ca<sup>2+</sup> environment, tilapia larvae not only increased Ca<sup>2+</sup> influx but also decreased Ca<sup>2+</sup> efflux, and thus resulted in an enhanced Ca<sup>2+</sup> net uptake. Developing larvae not only increased Ca<sup>2+</sup> influx but also decreased Ca<sup>2+</sup> efflux when they were acclimated to low-Ca<sup>2+</sup> environments. After acclimation for 8 d, influx and efflux of low-Ca<sup>2+</sup> group were about 106% and 43%, respectively, to those of high-Ca<sup>2+</sup> group. The enhanced calcium uptake capacity was characterized by 40-50 % decrease in K<sub>m</sub> and 7-25 % increase in J<sub>max</sub>. These results suggest that tilapia larvae are able to modulate their Ca<sup>2+</sup> uptake mechanism to maintain normal level of body Ca<sup>2+</sup> content and growth under the environment with different levels of Ca<sup>2+</sup>. Moreover, the sensitivity and response to low-Ca<sup>2+</sup> environments are age-dependent. Upon acute exposure to low Ca<sup>2+</sup>, newly hatched (H0) larvae increased both Ca<sup>2+</sup> influx (from 24% to 67% of high-Ca<sup>2+</sup>) and net uptake (from 5% to 69%) within 64 h, while 3-d-old (H3) larvae managed to reach the levels of the control within 38 h. Declining Ca<sup>2+</sup> efflux in H3 larvae occurred 14 h after exposure, much faster than did those in H0 larvae (38 h).

Table 1. Comparison of the kinetic parameters for  $\text{Ca}^{2+}$  influx in 3-d-old tilapia larvae reared in high- and low-calcium artificial freshwater

	Medium	$J_{\max}$ (pmol $\text{mg}^{-1} \text{h}^{-1}$ )	$K_m$ (mmol $\text{l}^{-1}$ )
Exp 1	High $\text{CaCl}_2$	201.9±5.1	0.0285±0.0024
	Low $\text{CaCl}_2$	251.8±7.8	0.0141±0.0014
Exp 2	High $\text{CaSO}_4$	228.5±6.0	0.0108±0.0008
	Low $\text{CaSO}_4$	244.1±5.4	0.0067±0.0001

Fertilized eggs were incubated in high- or low- $\text{Ca}^{2+}$  media until 3 days after hatching. Mean±SD (N=4) was indicated. Significant difference was found between low and high calcium groups in each experiment ( $t$  test,  $p<0.05$ )

### Comparisons among different species

Comparisons were made in the  $\text{Ca}^{2+}$  influx regulation among goldfish, zebrafish and ayu when acclimated to low- $\text{Ca}^{2+}$  environment, goldfish larvae show the best compensation in  $\text{Ca}^{2+}$  balance, a higher  $\text{Ca}^{2+}$  influx than that in control (high- $\text{Ca}^{2+}$ ), while ayu was the worst in compensation, about 74% decrease in  $\text{Ca}^{2+}$  influx. The species differences in the capacities for  $\text{Ca}^{2+}$  balance may be associated with the differences in the development patterns and the inhabiting environments of fishes.

### Comparison with adults

Compared with adults that merely maintain an almost constant level of internal  $\text{Ca}^{2+}$  content, developing larvae must have a continuous increase in body calcium content with development. The net  $\text{Ca}^{2+}$  uptake in developing larvae is about 2~7 fold higher than that in adults. The net uptake makes only a very minor, less than 0.01% of total content, contribution to increase the  $\text{Ca}^{2+}$  content of adult, which already have a considerable pool of  $\text{Ca}^{2+}$ . However, it provides a significant contribution (13~19%) to larval  $\text{Ca}^{2+}$  pool, which is still being developed. Moreover, developing larvae appear to regulate their  $\text{Ca}^{2+}$  balance more rapidly and efficiently upon environmental challenge, implying that developing larvae may allow much less fluctuation in the internal hydro-mineral conditions, which may be critical for the development and survival of larvae.

Table 2. Comparison of Ca<sup>2+</sup> balance between developing larva and adult of tilapia

	Adult	1-d-old larva	5-d-old larva
Body weight (mg)	20,000	6.7	9.5
Total content (nmole mg <sup>-1</sup> )	2×10 <sup>2</sup>	2.223	11.450
Influx (nmole mg <sup>-1</sup> h <sup>-1</sup> )	0.0279	0.0486	0.1572
Efflux (nmole mg <sup>-1</sup> h <sup>-1</sup> )	0.0081	0.0069	0.0133
Net flux (nmole mg <sup>-1</sup> h <sup>-1</sup> )	0.0198	0.0417	0.1439
(Net flux÷total content) ×100%	0.0099	18.76	12.57

Data of adult was from Flik et al. (1986).

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**OSMOREGULATION IN ADULT SEA TROUT FOLLOWING  
TRANSFER FROM SEA WATER TO FRESHWATER**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

**Introduction**

In sea trout, as in other anadromous salmonids, a well-known series of endocrine-controlled events (“smoltification”) occurs prior to downstream migration to the sea. These prepare the juvenile fish for marine osmoregulation before departure from freshwater. Research has concentrated particularly on biochemical events in the gills, such as induction of the enzyme  $\text{Na}^+$ ,  $\text{K}^+$ -activated ATPase, which plays a key role in the extrusion of chloride and sodium ions.

In view of the inverse osmo- and volume-regulatory problems faced by freshwater and marine teleosts, different mechanisms are required for maintenance of homeostasis in the two media. In addition to the running costs of these mechanisms, a not insignificant fraction of the fishes basal metabolism, there must be a certain cost involved in maintaining both sets of mechanism in readiness in those species which are frequently exposed to changes in environmental salinity (e.g. in estuaries). Teleosts which only migrate at certain fixed times in their life cycle may benefit energetically by going through a process of pre-adaptation (acquisition of the appropriate machinery for the environment to be entered) prior to migration. Another option, a period of gradual acclimation in the river mouth, exposes the congregated fish to predation risks.

The aim of the present study was to look for indications of changes in the freshwater osmoregulatory capacities of adult sea trout during and outside the periods when they would normally enter freshwater. The hypothesis being tested was that there was some pre-adaptation to hyperosmotic regulation (i.e. some kind of “reverse smoltification”) whilst the fish were still at sea.

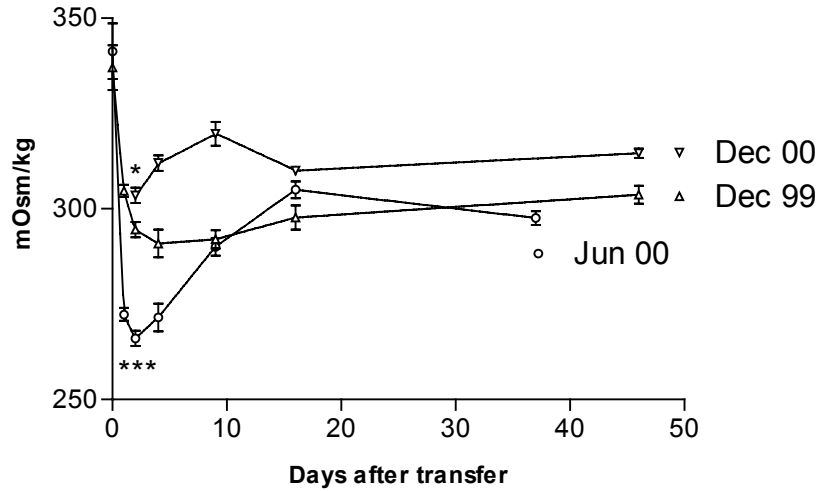
### **Materials and Methods**

Hatchery-reared sea trout (*Salmo trutta* L) were kept at a constant 10°C in a recirculating 24-28 ppt sea water system from smoltification in 1998 onwards. Groups were transferred directly to freshwater (at 10°C) in December 1999 and June 2000. Blood and gill biopsy samples were taken before and at intervals after transfer, under MS 222 anaesthesia. As this was obviously a highly stressful procedure a third group was transferred in December 2000 with fewer sampling points. No sampling was carried out until 2 days after transfer to freshwater (different individuals were sampled as the pre-transfer sea water point). Plasma osmolality was measured (Wescor Vapro 5520 vapor pressure osmometer) and diluted plasma analysed for inorganic cations and anions (Dionex 4500i ion chromatograph). Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase enzymatic activity was assayed at 25 °C using a coupled assay method. Messenger RNA was analysed with standard formaldehyde gel electrophoresis and Northern blotting. Species-specific <sup>32</sup>P-labelled cDNA probes were used for the quantification of Na<sup>+</sup>, K<sup>+</sup>-ATPase alpha-subunit and the secretory-type (NKCC1) Na<sup>+</sup>, K<sup>+</sup>, 2Cl<sup>-</sup> co-transporter mRNA abundance, using a β-actin probe to correct for loading differences. Band intensities were quantified using phosphor imaging (Molecular Dynamics, Sunnyvale, CA).

### **Results and Discussion**

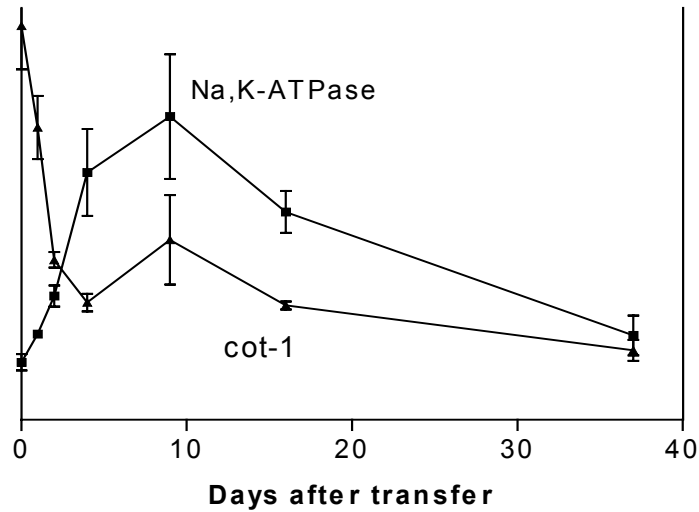
Plasma osmolality was around 340 mOsm kg<sup>-1</sup> in all groups before transfer. It fell following transfer (Fig. 1), reaching a minimum of 266 mOsm kg<sup>-1</sup> after 2 days in the June group (p< 0.001 cf. Dec. 1999 group). In the Dec.2000 group the 2 day (minimum) value was slightly higher (p<0.05) than the corresponding point in the Dec. 99 group and subsequent recovery was more rapid. By day 16 all groups had reached similar values.

**Fig. 1. Changes in Plasma Osmolality**



Na<sup>+</sup>, K<sup>+</sup>-ATPase activity varied a little during the the first 4 days after transfer and thereafter declined steadily to  $0.92 \pm 0.11 \mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$  (mean  $\pm$  SEM) by day 37 in the June group and to  $1.19 \pm 0.24 \mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$  by day 46 in the December 1999 group from initial sea-water values of  $3.27 \pm 0.26$  and  $4.06 \pm 0.29 \mu\text{mol ADP mg protein}^{-1} \text{h}^{-1}$  respectively. In contrast, expression of the Na<sup>+</sup>, K<sup>+</sup>-ATPase  $\alpha$ -subunit mRNA increased dramatically over the first 4 days after transfer in both June and December 1999 (Fig. 2, June group).

**Fig. 2 m-rna abundance (relative changes)**



Expression of the  $\text{Na}^+$ ,  $\text{K}^+$ ,  $2\text{Cl}^-$ -co-transporter cot-1 fell rapidly over the first 4 days in June and December (fig. 2, June group: Dec 1999 results were very similar). The contrast between the greatly increased expression of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase  $\alpha$ -subunit during the period in which the enzyme activity at first changed little and then started to fall was striking. Histological examination of the gill biopsy samples showed that this was during a period when typical freshwater chloride cells on the lamellae were replacing the seawater-type chloride cells in the filaments.

Sea trout transferred in December (when they would normally have migrated to freshwater) appeared to acclimate better to freshwater than those transferred in June (before the migration period). This raises the possibility that some pre-adaptation had already occurred.

**FRESHWATER ADAPTATION OF KILLIFISH INVOLVES  
MORPHOLOGICAL AND FUNCTIONAL ALTERATION IN  
BRANCHIAL CHLORIDE CELLS**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

The killifish, *Fundulus heteroclitus*, is a euryhaline species that can adapt to a wide range of salinities. Although chloride cells are more developed in seawater (SW) than in fresh water (FW) in most fishes examined to date (Uchida et al., 2000), it has been revealed that chloride cells in killifish are larger in FW than in SW. The apical membrane of branchial chloride cells in FW-adapted killifish showed projections with microvilli that expanded the apical surface area (Katoh et al., 2001), suggesting active ion uptake through chloride cells in FW. Thus, killifish is considered to serve as a suitable model for studies on chloride cell functions in FW. The model for active Na<sup>+</sup> uptake across the gill epithelia involves the vacuolar-type proton pump (V-ATPase) coupled with amiloride-sensitive Na<sup>+</sup> channel (Harvey and Wieczorek, 1997). In the present study, we investigated the effects of environmental Na<sup>+</sup> concentration on chloride cell morphology, and examined the implication of V-ATPase in Na<sup>+</sup> absorption through the gill epithelia in killifish.

## Materials and methods

Killifish of the Arasaki strain (Shimizu, 1997) were kept in a tank with recirculating SW at the ambient temperature. The fish were first adapted to 50% SW for 1 week, and then kept in normal FW for 1 month. The FW-adapted fish were separated into three groups and reared for 1 week in defined FWs with 0.1 mM, 1 mM, or 10 mM NaCl and 0.5 mM CaCl<sub>2</sub>. The fish were not fed and the water temperature was maintained at 25°C during the experiment. The gills were fixed and observed by confocal laser scanning microscopy (LSM), and scanning (SEM) and transmission (TEM) electron microscopy. Furthermore, we cloned and sequenced cDNA encoding A subunit of V-ATPase and examined immunolocalization of V-ATPase in the gill epithelia.

## Results

Plasma osmolality of the killifish adapted to defined FWs containing 0.1, 1 or 10 mM Na<sup>+</sup> was maintained within a physiological level, although there were significant differences among three experimental groups (P<0.01). There was no significant difference in gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity among three experimental groups (Table 1).

With decreasing environmental Na<sup>+</sup> concentration, chloride cells became significantly larger (Table 1), and extended their distribution toward the efferent-vascular side. In SEM observations, the apical membrane of chloride cells was located at the boundary of pavement cells. In 0.1 and 1 mM Na<sup>+</sup> groups, the apical membrane was equipped with microvilli on its surface. In 10 mM Na<sup>+</sup> group, on the other hand, the apical membrane of most chloride cells was invaginated to form an apical pit. The TEM observations showed that the chloride cells often formed multicellular complexes with accessory cells, and that chloride and accessory cells shared the apical pit in Na<sup>+</sup> 10 mM group. Such complexes were not observed in 0.1 and 1 mM Na<sup>+</sup> groups.

Table 1 Effects of ambient Na<sup>+</sup> concentration

	Na <sup>+</sup> 0.1 mM	Na <sup>+</sup> 1 mM	Na <sup>+</sup> 10 mM
Osmolality (mOsm kg <sup>-1</sup> )			
Gill Na <sup>+</sup> , K <sup>+</sup> -ATPase activity (μmol ADP mg protein <sup>-1</sup> h <sup>-1</sup> )	12.1±0.8 (8)	10.6±0.8 (8)	9.0±0.5 (7)
Gill chloride cells size (μm <sup>2</sup> )	201.8±5.9 (5)	117.7±4.2 (5)*	96.5±3.9 (5)*
density (cells mm <sup>-2</sup> )	3374±554 (5)	3774±235 (5)	4220±702 (5)

\*p<0.01 compared with Na<sup>+</sup> 0.1 mM group

†p<0.01 compared with Na<sup>+</sup> 1 mM group

A full length of cDNA encoding A subunit of V-ATPase (2573 bases) and the deduced amino acid sequence (618 amino acids) were obtained. These sequences showed high degrees of identity with V-ATPase from other animals. Immunocytochemistry with an antibody specific for killifish V-ATPase showed that the V-ATPase was distributed in the branchial chloride cells and pavement cells in FW-adapted fish, but the intensity of signal was faint in SW-adapted fish.

### Conclusions

The development and ultrastructural alternations of chloride cells in lower Na<sup>+</sup> environments suggest that chloride cells are the sites for Na<sup>+</sup> uptake in FW-adapted killifish. This is also supported by the immunolocalization of V-ATPase in the gill epithelia with the homologous antibody.

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**SEAWATER TRANSFER AND CONFINEMENT: EFFECTS ON  
BRANCHIAL NA-K-ATPASE  
ACTIVITY IN CHLORIDE AND PAVEMENT CELLS OF  
ATLANTIC SALMON SMOLTS.**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

In teleost fish the gills are the major organ involved in ion regulation and maintenance of hydromineral balance. This is essential given the hypo- and hyperosmolar conditions fish are facing in fresh and sea water respectively. This challenge is even more extreme for fish migrating between fresh and sea water like e.g. salmon. The general concept of ionregulation involves active accumulation of ions in freshwater with Na<sup>+</sup> absorption via the pavement cells. In seawater fish excrete ions mostly Na<sup>+</sup> and Cl<sup>-</sup> via the chloride cells. The Na-K-ATPase is the enzyme playing a crucial role in the functionality of these osmoregulatory cells. It is well established that cortisol, which in fish has both mineralo- and corticosteroid functions and its synthetic analogue dexamethasone play a crucial role in the modulation of Na-K-ATPase activity (Wendelaar Bonga, 1997). In addition it has been shown by means of catalytic histochemistry that dexamethasone regulates Na-K-ATPase activity differently in pavement and chloride cells of the European eel under fresh or sea water conditions respectively (Marsigliante et al., 2000). Cortisol in fish is the main endproduct of the hypothalamic-pituitary-interrenal axis which co-ordinates the stress response in fish (Wendelaar Bonga, 1997).

The aim of this study was to investigate the effects of net-confinement, an aquacultural relevant handling process, known to evoke a stress response in fish, hence resulting in increased plasma cortisol levels and seawater transfer (for 1 and 7 days) in combination with confinement, on gill Na-K-ATPase activity. In particular this study aimed to compare whole gill homogenate measurements of Na-K-ATPase activity with the catalytic histochemistry technique which specifically allows to study the distribution

Na-K-ATPase activity between chloride or the pavement cells in gill cryosections and also study expression of Na-K-ATPase related protein expression by means of immunohistochemistry using an antibody frequently used to determine fish gill Na-K-ATPase protein expression (Nolan et al., 1999; Wilson et al., 2000).

To determine whether confinement really induced a stress response in these fish plasma cortisol levels were measured by means of a radio immuno assay and indeed plasma cortisol levels in all groups of confined fish (day 0 = freshwater control, day 1 and 7 after sea water transfer) were significantly higher than plasma cortisol levels measured in fish sampled at rest. Whole gill homogenate Na-K-ATPase activity was increased in sea water fish when compared to fresh water animals and confinement resulted in an increase in enzyme activity in all groups. Catalytic histochemistry revealed that the confinement induced increase of Na-K-ATPase activity in the gills of freshwater fish was due to an increase in pavement cell related enzyme activity whereas in salmon transferred to seawater this increase in Na-K-ATPase activity was mostly observed in the chloride cells and this shift of enzyme activity from one cell type to the other progressed with duration of sea water transfer. Normal immunohistochemistry only revealed an increase in Na-K-ATPase related protein expression in chloride cells of seawater transferred fish, but under no conditions could any positive signal be detected in the pavement cells of any of the fish.

It is concluded that Atlantic salmon like European eel during seawater transfer shift Na-K-ATPase activity from the pavement to the chloride cells, which is in agreement with the two different roles attributed to these two cell types. The results obtained by means of normal immunohistochemistry, however, clearly show that this technique is not sufficient to describe and understand the mechanisms involved in seawater acclimatisation of fish.

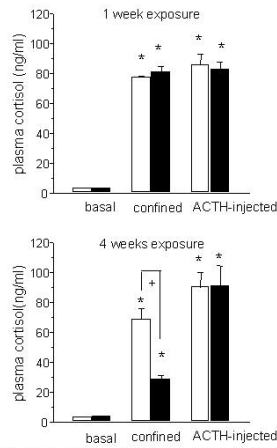


Figure 1: Effect of dietary E(α)P on the primary stress response in turbot. Data for trout and tilapia are published (Quelkus et al. 2000). Environmental Toxicology and Chemistry V ol 12 p 2892 - 2899. Open bars represent control fish and black bars represent fish receiving the E(α)P containing diet ( $n=8$  fish/SEM). Asterisks indicate significant differences from basal sample, and pluses indicate significant differences due to the E(α)P exposure.

Figure 1:FW gill Na-K-ATPase – effect of confinement. The upper picture shows the catalytic Na-K-ATPase activity in the gills of confined Salmon smolts, the lower picture shows the results obtained immunohistochemistry.

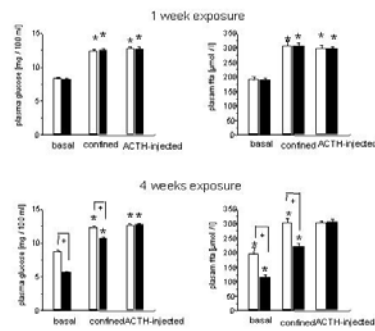
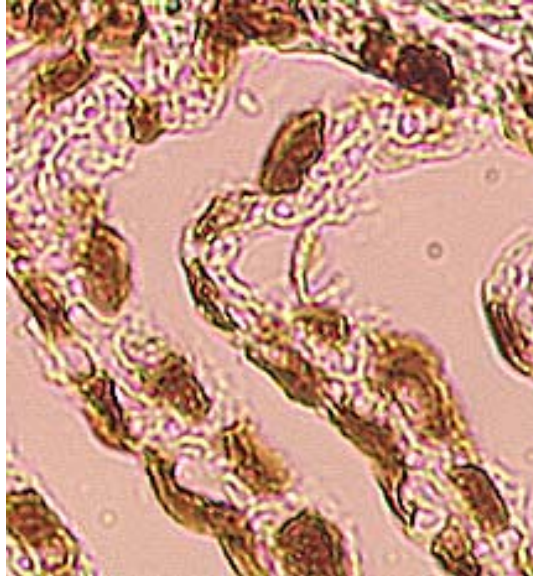


Figure 2: Effect of dietary B(a)P on secondary stress responses in haddock. Data for trout and tilapia are published (Quabius et al. 2000). Environmental Toxicology and Chemistry 19: 2592 - 2599. Open bars represent control fish and black bars represent fish receiving the B(a)P containing diet (n=3 *v.* SEM). Asterisks indicate significant differences from basal sample, and pluses indicate significant differences due to the B(a)P exposure.

Figure 2 :SW gill Na-K-ATPase – effect of confinement. The upper picture shows the catalytic Na-K-ATPase activity in the gills of confined Salmon smolts, the lower picture shows the results obtained immunohistochemistry

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**FUNCTIONAL DIFFERENTIATION OF CHLORIDE CELLS  
IN THE YOLK-SAC MEMBRANE  
OF MOZAMBIQUE TILAPIA EMBRYOS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Teleost fish maintain ion concentrations and osmolality of the body fluid at levels different from external environments. In adult fish, the gills, kidney and intestine are important osmoregulatory organs, creating ionic and osmotic gradients between the body fluid and external environments. In particular, gill chloride cells function as the salt-secreting site in seawater (SW) fish and probably as the ion-absorbing site in freshwater (FW) fish. In fish embryos and larvae, however, those osmoregulatory organs in adult fish are not yet developed or not fully functional. Nevertheless, embryos and larvae are also able to maintain ionic and osmotic gradients, and thus fish in early life stages should have other means of maintaining ion balance. In early life stages of fish when the gills are not yet developed, chloride cells are mainly distributed in the yolk-sac membrane, which covers the yolk, and the body surface (Kaneko et al., 2002). As the fish develop, the yolk is absorbed and, at the same time, the gills become functional. Thus, the functional site of chloride cells shifts from the yolk-sac membrane and body surface to the gills as the fish grow.

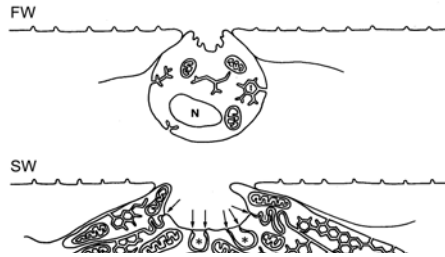


Figure 1. Freshwater (FW)- and seawater (SW)-type chloride cells in the yolk-sac membrane of tilapia embryos.

#### Occurrence of FW- and SW-type chloride cells

Mozambique tilapia *Oreochromis mossambicus* is a euryhaline teleost that is adaptable to a wide range of salinities from FW to SW. The tilapia can breed both in FW and in SW, and embryos and larvae can survive direct transfer from FW to SW, or *vice versa*. Taking advantage of the excellent euryhalinity of the tilapia, we examined morphological alteration of chloride cells in the yolk-sac membrane of the embryos and larvae in response to the environmental salinity.

In both FW and SW tilapia embryos and larvae, numerous chloride cells were detected in the yolk-sac membrane. However, chloride cells were much larger in SW fish than in FW fish. When FW-tilapia embryos were transferred to SW at the time of hatching, the chloride cell size increased significantly. Conversely, when SW embryos were transferred to FW at hatching, the chloride cell size decreased (Ayson et al., 1994). The enlarged chloride cells in SW formed multicellular complexes together with adjacent accessory cells, whereas chloride cells existed individually in FW (Figure 1; Shiraishi et al., 1997). Immunocytochemical studies showed that the CFTR Cl<sup>-</sup>-channel and Na<sup>+</sup>, K<sup>+</sup>, 2Cl<sup>-</sup>-cotransporter were located in the apical and basolateral membranes, respectively, in the SW-type chloride cell complexes, but not in the FW-type cells. Moreover, the chloride test and X-ray microanalysis revealed that the SW-type complexes had definitive function of Cl<sup>-</sup> secretion. These findings clearly indicate a significant role of chloride cells in the yolk-sac membrane in adaptation to SW.

### **Functional differentiation of chloride cells**

To examine the development of SW chloride cells, we observed *in vivo* sequential changes in chloride cell morphology during SW adaptation using a confocal laser scanning microscope (Hiroi et al., 1999). When transferred from FW to SW, single (FW-type) chloride cells enlarged and were indented by newly-differentiated accessory cells to form multicellular complexes (SW-type), suggesting plasticity in the ion-transporting functions of chloride cells. To further examine the functional differentiation of chloride cells, we developed a “yolk-ball” incubation system, in which the yolk sac was separated from the embryonic body and subjected to *in vitro* incubation (Shiraishi et al., 2001). The incision on the yolk ball was healed during incubation in balanced salt solution for 3 h, so that the yolk-sac membrane completely enclosed the yolk. The yolk balls prepared from FW-acclimated embryos were transferred either to FW or to SW, and incubated for up to 96 h. In the yolk balls transferred to SW, chloride cells often formed multicellular complexes, characteristic of SW-type chloride cells. In those transferred to FW, on the other hand, the cells were small and rarely formed a complex. The Cl<sup>-</sup>-turnover rate measured by the whole-body influx of <sup>36</sup>Cl<sup>-</sup> was about 60 times higher in the yolk balls in SW than in FW. Such responses of the yolk balls were identical to those observed in intact embryos.

These findings indicate that the chloride cells in the yolk-sac membrane are equipped with an autonomous mechanism of functional differentiation, independent of embryonic endocrine and nerve systems. The yolk-ball incubation system established here definitely serves as an excellent experimental model for further studies on chloride cell differentiation and functions.

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**SUB-TYPES OF MITOCHONDRIA RICH CELLS  
IN THE GILLS OF FRESHWATER RAINBOW TROUT**

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**Introduction:**

Rainbow trout are anadromous, existing in both freshwater and seawater environments. These environments cause opposing extremes relating to ionic loss and uptake for the trout. In a freshwater environment, the trout has a gradient of ion loss from its body to the hypo-osmotic external water. A fish living in a seawater environment has the reverse challenges as the medium has a higher concentration of ions than the fish (*i.e.* a fish in seawater will be constantly gaining ions by passive diffusion). The gill epithelium is a dynamic transporting membrane that manipulates ion exchange (*e.g.*, sodium and chloride) to achieve both ion and acid-base homeostasis. Classically, the gill epithelium is thought to consist of two cell types, the pavement cell, which makes up over 90% of the total gill area and the mitochondria-rich (MR) cells (Goss *et al.*, 1998). It is believed that the MR cell is the major regulation site for ion balance (Goss *et al.*, 1992; Perry, 1998).

**Results and Discussion**

Recently, our lab has developed a method of separating the MR cells into at least two functionally distinct populations of cells (Goss *et al.*, 2001; Galvez *et al.*, 2002, Reid *et al.*, submitted). We have demonstrated that peanut lectin agglutinin (PNA) binds only to one sub-type of the MR cells of the fish gill (Goss *et al.*, 2001), which we term PNA-positive MR cells (PNA<sup>+</sup>). A magnetic bead separation system was developed to isolate the PNA<sup>+</sup> MR cells from the PNA negative (PNA<sup>-</sup>) MR cells (Galvez *et al.*, 2002). Separation allowed for morphological and physiological characterization of each of the cell types. While both cell types are rich in mitochondria, PNA<sup>+</sup> cells have an ovoid

nucleus with a dense vesiculo-tubular network while PNA<sup>-</sup> cells have distinct morphological features including irregular nuclei, dense peripheral chromatin, and a less developed vesiculo-tubular network. Furthermore, we have demonstrated that acid-activated and phenamil-sensitive Na<sup>+</sup> uptake, an indicator of eNaC mediated Na<sup>+</sup> uptake, as well as environmentally induced alterations in H<sup>+</sup> ATPase expression and activity, are found only on PNA<sup>-</sup> MR cells. We propose updating the current model for gill ion transport in the cells of freshwater fish (see Figure 1) linking Na<sup>+</sup> uptake to an electrogenically coupled H<sup>+</sup> ATPase on PNA<sup>-</sup> (termed  $\alpha$ -type) MR cells type and suggest that chloride uptake/base exchange is mediated by PNA<sup>+</sup> (termed  $\beta$ -type) MR cells.

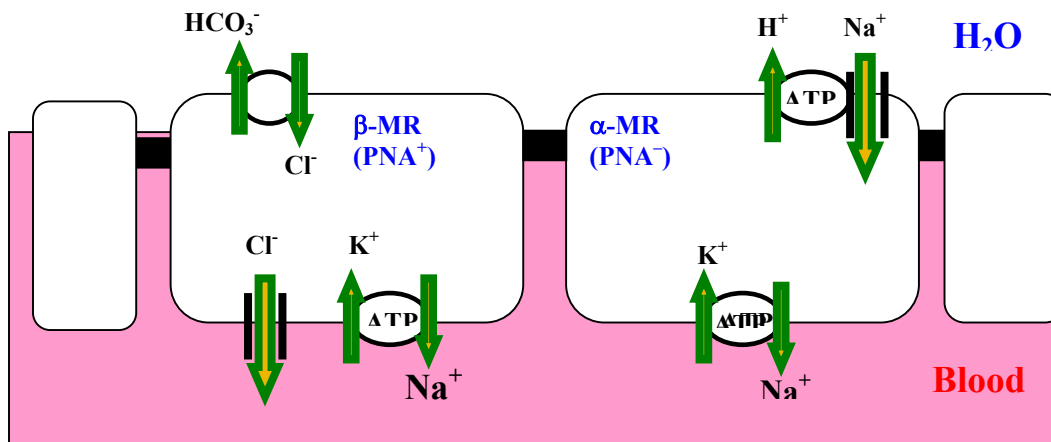
### **Acknowledgements**

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Figure 1. Proposed Freshwater Fish Gill Ion Transport Model:





**CHANGES IN THE SURFACE ULTRASTRUCTURES OF  
MITOCHONDRIA-RICH CELLS AND EXPRESSION OF SODIUM  
PUMPS IN GILLS OF TILAPIA, *OREOCHROMIS MOSSAMBICUS*,  
TRANSFERRED FROM SEAWATER TO FRESH WATER**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

Mozambique tilapia (*Oreochromis mossambicus*) is able to maintain the osmotic constancy of internal milieu and survive in hypertonic salt water or hypotonic fresh water (Hwang et al. 1989). Biochemical studies on long-term adaptation showed that activities as well as contents of gill Na,K-ATPase in tilapia changed with environmental salinity (Lee et al., 2000). Moreover, ultrastructures of gill epithelial mitochondria-rich (MR) cells where most gill sodium pump was located were found to change with external salinity (Lee et al., 1996; Lee et al. 2000). Being a freshwater euryhaline species, Mozambique tilapia cannot survive after direct transfer from freshwater (FW) to seawater (35‰, SW), unless pre-adapted to 20‰ salt water (BW) for at least 24hrs. However, tilapia adapt rapidly after direct transfer from SW to FW (Hwang et al. 1989). To realize the mechanism of FW-adaptation in SW tilapia, a series of time course experiments were performed. Gills were sampled, phenotypic changes of MR cells were examined, and expression of Na,K-ATPase from abundance of  $\alpha$ -subunit mRNA and protein to activity were compared.

**Experimental designs and results**

Mozambique tilapia reared in SW were transferred directly to FW and

sampled at 0, 3, 6, 12, 24, 48hrs, and 30 days. Serum was collected for analysis of osmolality and gills were dissected out for ultrastructural and biochemical and molecular examination.

Within 3hrs after transfer, a significant decrease in serum osmolality was found. After 3hrs, the osmolality declined slowly with time until the osmolality of the 48hrs group reached the same level as the 1-month (FW) group. For expression of branchial sodium pump in three different levels, i.e., mRNA, protein, and activity, significant decrease occurred in 6hrs, 24hrs, and 3hrs post-transfer, respectively (Fig. 1). No significant changes in branchial MR cell density were found in tilapia transferred directly from SW to FW (Fig. 2).

There is only one type (deep hole) of MR cells in SW tilapia. However, the vicissitudes of MR cell phenotypes (wavy convex, shallow basin, and deep hole) occurred within 48hrs after transfer to FW (Fig. 2).

Our results showed that the surface ultrastructures of MR cells change from SW type to FW type during 48hrs after transfer. Moreover, the activity, protein and mRNA abundance of gill sodium pumps as well as serum osmolality come to stable and exhibit similar levels of freshwater fish in 48hrs post-transfer. It is concluded that it takes two days for tilapia to adapt from SW to FW.

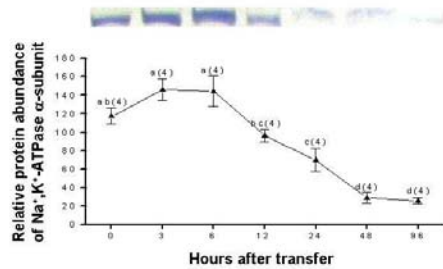


Figure 1. Changes of Western blot and relative protein abundance of Na<sup>+</sup>, K<sup>+</sup>-ATPase α-subunit of tilapia gill following transfer from 35‰ SW to FW. Significant decrease occurred within 24hr after transferring to FW

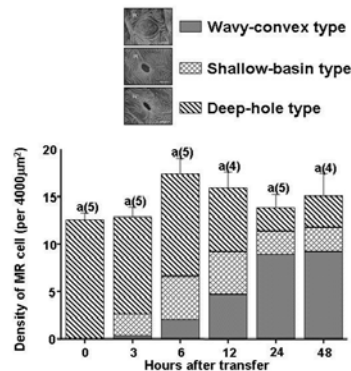


Figure 2. Changes in density and type of MR cell of tilapia gill filament following transfer from 35‰ SW to FW. No difference of MR cell density was found. Wavy-convex type of MR cell showed significant increase after transferring to FW.

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**IMMUNOCYTOCHEMICAL TRACKING OF CFTR AND NKCC  
TRANSPORTERS IN CHLORIDE CELLS AND INTESTINE OF  
KILLIFISH (*FUNDULUS HETEROCLITUS*):  
CHANGES WITH SALINITY ADAPTATION**

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**EXTENDED ABSTRACT ONLY - PLEASE DO NOT CITE**

The gill epithelia of teleost fish secrete NaCl in seawater (SW) and absorb NaCl in freshwater (FW). Na<sup>+</sup>,K<sup>+</sup>-ATPase pump in both salinities provides the driving force, but it is the placement of passive transporters that provides the direction. In SW a basolaterally located Na<sup>+</sup>,K<sup>+</sup>,2Cl<sup>-</sup> cotransporter (NKCC) transports Cl<sup>-</sup> into the ion secreting cell. At the apical membrane is an anion channel that is a homolog for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) that allows Cl<sup>-</sup> exit (review: Marshall 2002). Killifish CFTR (kfCFTR) has been cloned from the euryhaline teleost fish *Fundulus heteroclitus* and this protein is expressed in gill, opercular epithelium and intestine, as detected by Northern blot analysis (Singer et al. 1998). CFTR, detected immunocytochemically, is restricted to the membrane in apical crypts of chloride cells of seawater adapted mudskippers (Wilson et al. 2000). In salinity adaptation from FW to SW, ion transport direction reverses from uptake to secretion, but it is not clear how this reversal occurs. In linear time courses of salinity adaptation in tilapia (*Oreochromis mossambicus*) larvae (Hiroi et al. 1999), more than 70% of the same chloride cells are still present 96h after transfer to sea water, so the redistribution of transporters in extant cells is an important component of adaptation. This study traces, using immunocytochemistry, NKCC and CFTR in chloride cells during SW adaptation.

## Methods

Opercular membranes containing mitochondria rich chloride cells were dissected and stained with Mitotracker Red (Molecular Probes), then fixed (80% MeOH/20% DMSO) and incubated with primary antibodies, mouse monoclonal anti hCFTR carboxy terminus (R&D systems) and mouse monoclonal anti hNKCC (T4, Iowa Hybridoma Bank). The secondary antibody was goat anti mouse Oregon Green 488 (Molecular Probes). Whole opercular membranes were viewed with a confocal microscope (Olympus FV300).

## Opercular Membrane CFTR Immunofluorescence

Cellular distribution of CFTR immunofluorescence was observed in chloride cells of FW adapted fish and animals transferred to SW for 24h, 48h and 14+ days. Confocal microscopy allowed localization within mitochondria rich (MR) cells to be determined as superficial (in the membrane of the apical crypt) or in the basolateral membrane of the cells. In FW, 90 percent of MR cells had diffuse kfCFTR immunofluorescence in the central part of the cell (Figure 1B), with only 8.1 percent having apical kfCFTR that was  $6.6 \pm 0.54$  (mean  $\pm$  SEM)  $\mu\text{m}$  below the microridges of surrounding pavement cells. Curiously, pavement cells from FW (but not SW) killifish had positive immunofluorescence for kfCFTR, suggesting that pavement cells contribute to ion uptake in FW. After 24h in SW, a time when kfCFTR expression is elevated (Singer et al. 1998), there appeared among 18.8 percent of MR cells, a

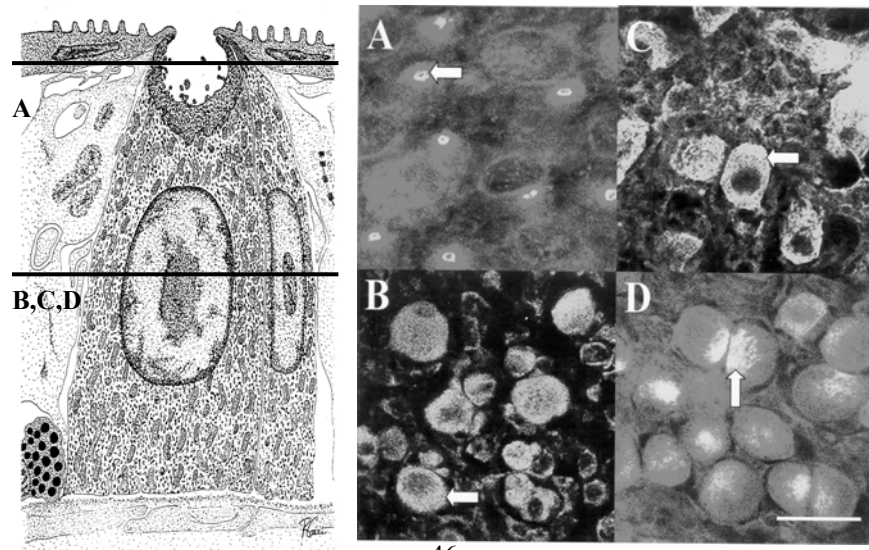


Figure 1. LEFT Drawing of chloride cell showing planes of optical sections. RIGHT Immunocytochemistry of opercular membranes (white; arrows) and mitotracker stain (Grey). A) CFTR apical distribution in SW. B) CFTR diffuse basolateral distribution in FW. C) NKCC diffuse basolateral distribution in SW. D) Eccentric NKCC distribution in FW. Bar is 20  $\mu\text{m}$ . (fm. Marshall et al 2002)

condensed punctate immunofluorescence that was  $13.4 \pm 0.66 \mu\text{m}$  below the surface of the cells. By 48h a majority (76.3%) of MR cells had punctate kfCFTR distribution and the distance from the surface was less ( $7.8 \pm 0.2 \mu\text{m}$ ), a distribution approaching the SW acclimated condition. Often the apical membrane CFTR immunofluorescence was identifiable as the characteristic ring at the apex of the cell (Figure 1A). In SW acclimated fish all MR cells had the ring shape kfCFTR immunofluorescence that was  $6.1 \pm 0.04 \mu\text{m}$  below the surface. Thus CFTR, the anion channel responsible for  $\text{Cl}^-$  secretion in marine teleosts, redistributes in MR cells during SW acclimation by condensation of a diffuse distribution below the apical crypt followed by translocation and insertion in the apical membrane.

#### **Opercular membrane NKCC Immunofluorescence**

Unlike the CFTR distribution in FW, NKCC immunofluorescence was condensed and localized in lateral parts of MR cell complexes in FW animals (Figure 1D). Where chloride cells were paired, NKCC immunofluorescence appeared where the two cells abutted. NKCC redistributed to the whole basal cytoplasm after acclimation to SW (Figure 1C). Because SW ion secretion is blocked by basolateral furosemide and bumetanide, the NKCC fluorescence is very likely in the tubular system, not the cytosol. NKCC, the cotransporter that translocates  $\text{Cl}^-$  across the basolateral membrane, moves from an eccentric cytosolic location in fresh water to a diffuse basolateral localization in SW chloride cells.

#### **Intestine NKCC and CFTR immunofluorescence**

CFTR was immunocytochemically localized in intestine frozen sections and found to be present in basolateral membranes of all enterocytes and in the brush border membrane of some (approx. 25%) cells. In contrast, NKCC immunofluorescence was in the basolateral and brush border membranes of most enterocytes and in the basolateral membrane only in a minority (approx. 25%) of cells. Sections of killifish posterior intestine were induced to secrete

NaCl and fluid by the calcium ionophore ionomycin (1.0  $\mu$ M) in combination with agents to elevate intracellular cyclic AMP, dibutyryl-cAMP (db-cAMP) 0.5 mM with 0.1 mM 3-isobutyl-1-methylxanthine (IBMX). Mucosal application of the anion channel blocker 1.0 mM diphenylamine-2-carboxylate (DPC) after ionomycin + db-cAMP + IBMX significantly reduced serosal to mucosal unidirectional Cl<sup>-</sup> flux ( $P < 0.001$ ), net Cl<sup>-</sup> flux ( $P < 0.05$ ), short circuit current ( $I_{sc}$ ,  $P < 0.001$ ) and tissue conductance ( $G_t$ ,  $P < 0.001$ ), while 0.1 mM of the disulfonic stilbene DIDS was without effect. Teleost intestine is capable of salt and fluid secretion if intracellular Ca<sup>2+</sup> and cAMP pathways are stimulated together. The ion and fluid secretion appears to involve activation of CFTR ion channels in the apical membrane of a subpopulation of enterocytes.

### Conclusions

- 1) The anion channel CFTR redistributes from the basolateral membrane of chloride cells in FW to the apical crypt membrane during SW adaptation.
- 2) The cotransporter NKCC moves from an punctate locus eccentric to the nucleus to a diffuse basolateral location during SW adaptation.
- 3) The teleost chloride cell may be an ideal model to study trafficking of ion transport proteins in epithelial cells.

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### **Acknowledgements**

This work was funded by NSERC research grant to W.S.M. and USRA scholarship to J.A.H. and confocal microscope was purchased by CFI and ACOA.



**SODIUM UPTAKE KINETICS AND RESPONSES TO HIGH pH IN  
FISH INHABITING A SEASONALLY ALKALINE LAKE**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

The fish used in this study inhabit a shallow freshwater lake (Slapton Ley, Devon, UK), which has suffered on-going cultural eutrophication since the 1940s. As a consequence blooms of cyanobacteria occur in most summers, causing deterioration of water quality, namely a rise in water pH associated with accelerated algal photosynthesis (Halstead and Tash, 1982). The average pH of Slapton Ley ranged between 9.2 and 9.5 (max pH 10.1) in the summers of 2000 and 2001. Little is known of the physiology of the fish species native to Slapton Ley (perch, pike, roach and rudd), and the pHs (> 9.0) recorded in Slapton are in the range seen to have severe effects on rainbow trout physiology. These physiological disturbances include a drop in plasma electrolytes (Na<sup>+</sup> and Cl<sup>-</sup>), associated with a reduction in ion uptake rates (Laurent et al., 2000), and inhibition of ammonia excretion attributed to reversal of the NH<sub>3</sub> gradient across the gill (Wilkie, 1997). In order to understand the function of ionic transport systems it is important to analyse their transport kinetics (Shaw, 1959). This can be done by measuring the transport rate at a variety of substrate (external ion) concentrations, and can reveal possible differences in ionoregulatory strategies that may be correlated to an animal's environment and lifestyle (Gonzalez and Wilson, 2001). The aim of this study was to investigate the relative tolerances of the Slapton species to alkaline water, with respect to ion regulation and ammonia excretion disruption, as well as characterising their ion uptake mechanisms as a potential means to explain any tolerance.

**Materials and Methods**

Perch (*Perca fluviatilis*), pike (*Esox lucius*), roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*) were obtained from Slapton Ley by electrofishing, and rainbow trout (*Oncorhynchus mykiss*) from a local trout farm. Animals were held in dechlorinated Exeter tap water ([Ca<sup>2+</sup>] ~ 600.5

$\mu\text{mol l}^{-1}$ ;  $[\text{Na}^+] \sim 437.3 \mu\text{mol l}^{-1}$ ; TAlk  $\sim 0.90 \text{ mmol l}^{-1}$ ;  $10.7^\circ\text{C}$ ; pH 7.4) and withheld food in the week prior to an experiment. Experiments were carried out with individual fish held in flux chambers of varying volumes of aerated water. Fish were exposed to high pH ( $9.5 \pm 0.1$ ; using KOH) for 1 h, and increasing external  $[\text{Na}^+]$  (using NaCl) for 0.75 h in order to analyse  $\text{Na}^+$  kinetics. In both experiments sodium influxes ( $J_{\text{Na}}^{\text{in}}$ ) were measured by monitoring the disappearance of  $^{22}\text{Na}$  radioisotope from the external medium. Net sodium fluxes ( $J_{\text{Na}}^{\text{net}}$ ) were calculated from changes in total  $[\text{Na}^+]$  measured by flame photometry, and effluxes ( $J_{\text{Na}}^{\text{out}}$ ) were calculated as the difference between net flux and influx. Ammonia excretion was determined by its increase in the external medium, measured by colourimetric assay. Kinetic parameters ( $K_m$  and  $V_{\text{max}}$ ) were determined for individual fish using enzyme kinetics software (GraFit). All values are expressed as means  $\pm$  standard error. Negative and positive flux values indicate a loss from or gain to the fish, respectively, and were compared using repeated measures ANOVA ( $P \leq 0.05$ ) with each fish as its own control.

## Results

Acute exposure to pH 9.5 water caused significant changes to the  $\text{Na}^+$  fluxes of all species (Figure 1). Perch and roach showed significantly more negative  $J_{\text{Na}}^{\text{net}}$  during high pH exposure associated with an increased  $J_{\text{Na}}^{\text{out}}$ , only perch showed recovery of  $J_{\text{Na}}^{\text{net}}$ . Rudd and trout displayed no change in  $J_{\text{Na}}^{\text{net}}$  throughout the experiment, and both species had reduced  $J_{\text{Na}}^{\text{in}}$  and  $J_{\text{Na}}^{\text{out}}$  in the recovery period. Trout showed elevated  $J_{\text{Na}}^{\text{in}}$  and  $J_{\text{Na}}^{\text{out}}$  during high pH exposure. Sodium uptake in all four species displayed typical saturation kinetics, and  $K_m$  and  $V_{\text{max}}$  varied between the different species (Table 1). All species (including pike) showed similar inhibition of ammonia excretion during alkaline exposure (40-60%).

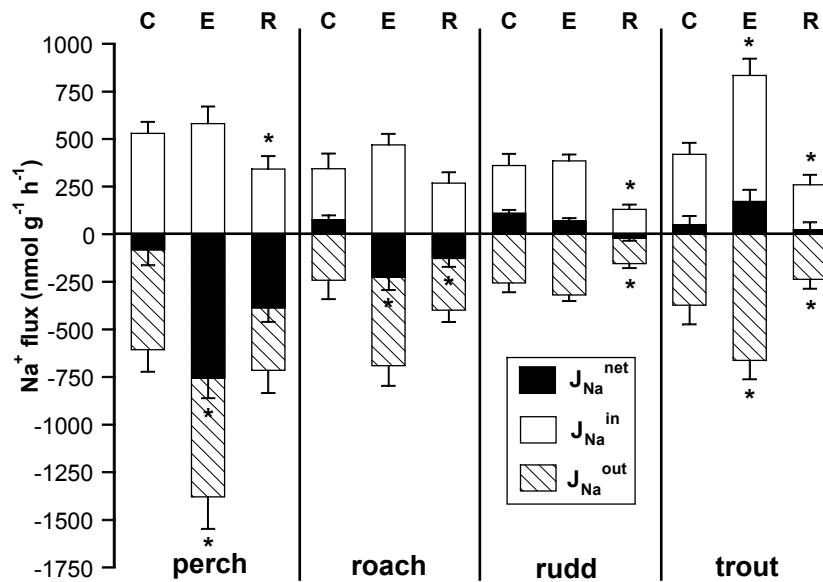


Figure 1. Effect of pH 9.5 water on the Na<sup>+</sup> fluxes of 3 Slapton fish species and rainbow trout. C = control flux (pH 7.4), E = experimental flux (pH 9.5) and R = recovery flux (pH 7.4)

Table 1. Mean K<sub>m</sub> and V<sub>max</sub> values for each species.

	K <sub>m</sub> (μmol l <sup>-1</sup> )	V <sub>max</sub> (nmol g <sup>-1</sup> h <sup>-1</sup> )
perch	69.2 ± 13.4	645.3 ± 83.9
roach	190.2 ± 56.9	713.7 ± 124.7
rudd	465.5 ± 73.8	832.4 ± 83.5
rainbow trout	163.7 ± 37.6	618.6 ± 174.7

### Conclusions

Short term exposure to pH 9.5 had no effect on the net Na<sup>+</sup> balance of rainbow trout, and caused increased Na<sup>+</sup> uptake. There may be a varying degree of tolerance to high pH water with regard to Na<sup>+</sup> balance in the Slapton species, with perch being the least tolerant, followed by roach and rudd. Varying Na<sup>+</sup> uptake characteristics were also recorded, with the fish most susceptible to Na<sup>+</sup> loss (perch) having the highest affinity transporter

and vice versa. The effect that high pH exposure had on ammonia excretion was markedly similar in all species.

### **Acknowledgements**

This work has been sponsored by The Whitely Wildlife Conservation Trust, South West Water, English Nature and The Environment Agency. Many thanks also to the staff at the Slapton Ley Field Centre and all who have assisted with this project.

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**AN ANALYSIS OF AQUAPORIN CHANNELS AND THEIR  
POTENTIAL ROLES IN OSMOREGULATION IN  
EELS AND OTHER TELEOST FISH.**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

To date, thirteen aquaporin water and small solute channel isoforms have been identified in mammals (Ishibashi *et al.*, 2000; Hatakeyama *et al.*, 2001). However, in vertebrates, one of a few notable absences from the list of species known to possess these small membrane proteins, was until recently, the teleost fish. The presence of aquaporin gene homologues in teleost fish was first described in Cutler *et al.*, (2000), but recently further information has become available on the role of aquaporins in this phylogenetic group (Cutler *et al.*, 2001; Virkki *et al.*, 2001). However with the recent release of the draft sequence of the pufferfish (*Fugu*) genome (October 2001) it has become possible to identify further aquaporin homologues in the teleost genome, and to compare and review this additional information with that already available.

**Aquaporins in the Pufferfish Genome.**

The current pufferfish draft genome contains 13 genes sharing homology with aquaporins (Table 1.). Amongst these, pufferfish appear to possess orthologous copies of AQP0, 1, 3, 4, 7 and 8, as well as apparent duplicate copies of AQP0 and 1 (which may have arisen from an ancient genome duplication event; Cutler *et al.*, 2001). In addition, there are also genes which share sequence homology to aquaporins but which are not sufficiently similar to individual isoforms to be described as direct orthologues. One of these is most similar to the sub-group of

aquaporins which are generally recognised to be solely water permeable (aquaporins 0, 1, 2, 4, 5, 6), the other four, belong to the glycerol-aquaporin sub-group (which are additionally known to be able to transport urea and glycerol).

Type	Mammalian Nomenclature	<i>Fugu</i> Orthologues	Eel (and Other Teleosts) Orthologues
<b>Aquaporins</b>	AQP1	AQP1 AQP1 Duplicate	AQP1 AQP1 Duplicate
	AQP2	-	-
	AQP0	AQP0 AQP0 Duplicate	AQP0 ( <i>Fundulus</i> ) -
	AQP5	-	-
	AQP4	AQP4	-
	AQP6	-	-
		AQP?	-
<b>Glycero-Aquaporins</b>	AQP7	AQP7	-
	AQP3	AQP3	AQP3 (and <i>Tribolodon</i> )
	AQP10	-	-
	AQP9	-	-
		AQP? AQP?	AQP? -
		AQP? AQP?	- -
<b>Divergent Aquaporins</b>	AQP8	AQP8	AQP8
	AQPX1	-	AQPX1?
	AQX2	-	-

Table 1. Nomenclature of aquaporin channels, in mammals, as well as in pufferfish (*Fugu*), eel (*Anguilla*) and other teleost species.

### **Water Transport in Osmoregulation.**

In teleost fish, there are several potential roles for water transporters in osmoregulatory processes. These include the absorption of water across the intestine following drinking in marine teleosts, absorption/loss of water across the gills, and the concomitant re-absorption of water together with ions in the kidney of freshwater fish.

### **Aquaporin3 in Eel Gill.**

The object of this study was to identify aquaporins within osmoregulatory tissues of the eel, and characterise any changes in their gene expression associated with salinity acclimation. Initial experiments led to the isolation of an AQP3 orthologue; its mRNA is expressed at high levels in the gill with lower levels in the eye, oesophagus and intestine. Expression experiments using yellow or silver eels showed that branchial AQP3 mRNA abundance decreased to 3% (of freshwater values) in seawater acclimated fish. Using an eel AQP3 antibody, the level of branchial AQP3 protein abundance was also shown to be 3-fold lower in seawater acclimated eels, and localisation studies identified chloride cells as a major site of gill AQP3 expression. The data suggests that AQP3 may play a role in similar changes in gill osmotic water permeability previously demonstrated.

### **Aquaporin1 in Eel Intestine.**

Subsequent experiments identified an orthologue of AQP1 expressed in the intestine, but some mRNA expression was also found in oesophagus, gill and kidney and other tissues. Initial quantitative studies revealed that the level of intestinal AQP1 mRNA expression increased 10-25x following seawater transfer, in contrast mRNA abundance decreased by 72% in yellow eel kidney. The high level of AQP1 mRNA expression in the intestine of marine eels suggests AQP1 may play a role in water absorption in this tissue.

### **Aquaporins in Eel Kidney.**

Further experiments with eel kidney led to the discovery of AQP1 dup, which is a duplicate copy of AQP1. AQP1 dup mRNA expression was only identified in the intestine, oesophagus and kidney. The level of AQP1 dup kidney mRNA expression decreased following seawater transfer of eels. The presence of higher

levels of expression of both the AQP1 and AQP1 dup isoforms in freshwater eels suggests that they may play a role in the re-absorption of water associated with salt retention in this environment, although other roles in other cells types can by no means be excluded.

#### **Aquaporins in Eel Oesophagus.**

The presence of expression of AQP3, AQP1 and AQP1 dup in the oesophagus was on the face of it somewhat puzzling as this tissue is particularly impermeable in marine fish, with only a small net osmotic water movement across this epithelium. However, as uni-direction fluxes have not been measured, one possibility is that there is a balance in the oesophagus between the absorption of water (and ions), with an opposing and nearly equivalent loss of water by osmosis to the imbibed seawater in the oesophageal lumen. The aquaporin channels present in this tissue may play a role in either of these opposing water transport phenomena.

#### **Other Aquaporins.**

Data on the characteristics of the other three eel aquaporin homologues also isolated (see Table 1.) will also be presented if at all possible.

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### **Acknowledgements**

The authors would like to acknowledge and thank the Natural Environment Research Council who have provided the funding for this work



**EFFECT OF SALINITY ON NA,K-ATPASE AND NA,K,2CL-  
COTRANSPORTER IN THREE SALMONIDS WITH VARYING  
DEGREES OF SALINITY TOLERANCE**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Chloride cells in the gills of teleosts are the major site of ionic regulation. We compared seawater tolerance and chloride cell morphology of three salmonids, lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*). All fish were one-year old juveniles and experiments were conducted in the spring. Following direct transfer from 0 ppt to 30 ppt, 80% of lake trout, 50% of brook trout and 100% of Atlantic salmon survived for 1 week. Plasma Na, K and Cl levels increased rapidly and remained high in lake trout, increased rapidly but were restored by 1 week in brook trout, and no significant change was observed in Atlantic salmon, indicating excellent seawater tolerance that is characteristic of smolts.

These three species were then transferred from 0 ppt to 10 ppt, 20 ppt and 30 ppt at 1-week intervals to examine gill Na,K-ATPase, Na,K,2Cl-cotransporter and chloride cell morphology. During the transfer, little mortality was observed and plasma ion levels remained stable in brook trout and Atlantic salmon. However, 20% of lake trout died and plasma ion levels increased after transfer to 30 ppt. In lake trout and brook trout, gill Na,K-ATPase activity was low in 0 ppt and increased 2.5 fold after 3 weeks in 30 ppt. Gill Na,K-ATPase activity of Atlantic salmon was already high in 0 ppt and remained high after the transfer. Western blotting revealed that gill Na,K,2Cl-cotransporter abundance in lake trout and brook trout was low in 0 ppt and increased 5-20 fold after the transfer, and that of Atlantic salmon was already high in 0 ppt and increased 1.5 fold after the transfer.

These results indicate a direct role of Na,K-ATPase and Na,K,2Cl-cotransporter in seawater acclimation.

Na,K-ATPase and Na,K,2Cl-cotransporter were co-localized to gill chloride cells on both the primary filaments and secondary lamellae in all three species in 0 ppt. After seawater transfer, both the size and staining intensity of chloride cells increased in all three species, but the distributional pattern of the cells varied among the species: chloride cells were still distributed in both filaments and lamellae in lake trout and brook trout; whereas, lamellar chloride cells of Atlantic salmon decreased in number and finally disappeared. Since the disappearance of lamellar chloride cells after seawater transfer has not only been observed in Atlantic salmon but also in other species (e.g. chum salmon *Onchorhynchus keta*, Japanese eel *Anguilla japonica*, Japanese sea bass *Lateolabrax japonicus* and Atlantic shad *Alosa sapidissima*), filament and lamellar chloride cells have been considered to be involved in seawater and freshwater acclimation, respectively. However, our results in lake trout and brook trout suggest that lamellar chloride cells could be also functional during seawater acclimation.

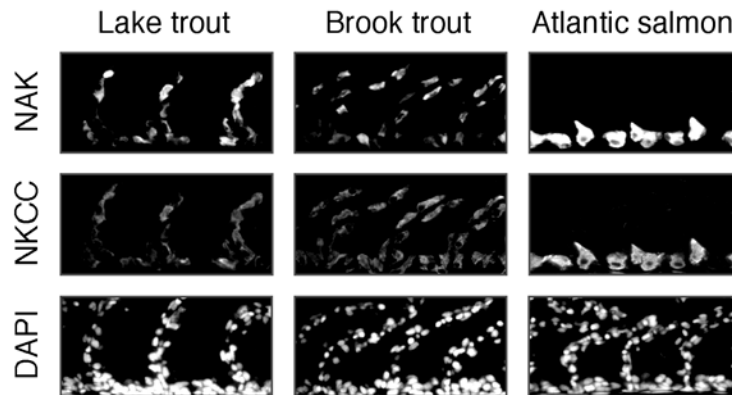


Figure 1. Immunofluorescence images of chloride cells in the gills of lake trout, brook trout and Atlantic salmon acclimated to 30 ppt seawater for 3 weeks. NAK-, Na, K-ATPase; NKCC, Na,K,2Cl-cotransporter; DAPI, counter staining using DAPI.

**THE CONSEQUENCES OF INTESTINAL BICARBONATE  
SECRETION ON ION, OSMOTIC AND ACID-BASE REGULATION  
IN THE EURYHALINE EUROPEAN FLOUNDER**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

Marine teleost fish are hypo-osmotic to the medium they inhabit. To avoid dehydration they drink the external seawater, absorb the majority of the imbibed volume within the intestine, and the remainder is excreted as rectal fluid. However, the rectal fluid is both alkaline (pH 8.4-9.0) and rich in the basic  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  ions (50-150 mM; Walsh et al., 1991; Wilson et al., 1996; 1999). This bicarbonate-rich environment causes the precipitation of imbibed calcium and magnesium as insoluble carbonates that are subsequently excreted along with the rectal fluids. The removal of these divalent cations by precipitation possibly serves two important functions: 1) to minimise the absorption of calcium (and its subsequent costly excretion via the kidney), and 2) to reduce the osmolality of intestinal fluid thus promoting water absorption into the blood. The importance of these roles is suggested by the linear increase of intestinal bicarbonate secretion with external salinity (and hence with drinking rate and passage of divalent ions into the intestine). Changes in intestinal bicarbonate secretion rates (i.e. net base excretion) will presumably have additional consequences for whole animal acid-base balance. The aim of the present study was to quantify how changes in bicarbonate secretion and calcium precipitation within the intestine impact a) the uptake of calcium and water from the intestine, and b) whole animal acid-base balance.

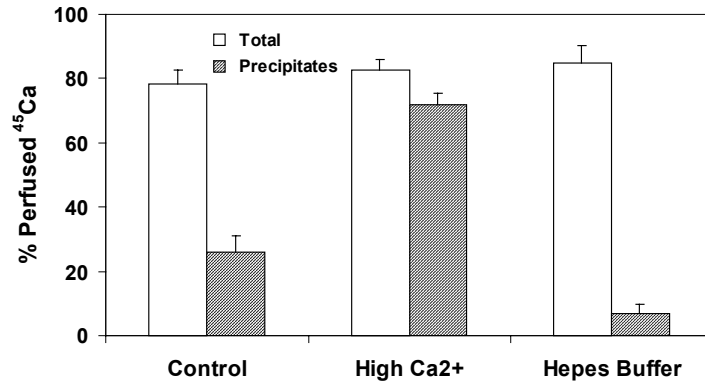
## Materials and Methods

European flounder (*Platichthys flesus*), were obtained from commercial fishing in the northern part of the Sound of Copenhagen, Denmark, and held in a re-circulating artificial seawater system at  $13\pm 1^\circ\text{C}$ . Fish were then prepared for *in situ* perfusion of the intestine according to the method of Grosell *et al.* (1999). Following surgery flounder ( $n=21$ ) were placed in individual flux chambers and the intestine perfused at  $\sim 3.8 \text{ ml kg}^{-1} \text{ h}^{-1}$  with one of 3 different gut salines designed to stimulate or decrease bicarbonate secretion and/or calcium precipitation; 1) Control saline (with  $5 \text{ mM Ca}^{2+}$ ), 2) High calcium saline ( $20 \text{ mM Ca}^{2+}$ ), 3) Buffered Saline ( $5 \text{ mM Hepes}$ ,  $\text{pH } 7.5$ ), all with matched osmolality ( $310 \text{ mOsm kg}^{-1}$ ). Rectal fluids were continuously collected via catheters during 72 h of perfusion. Salines also contained  $^{45}\text{Ca}$  to trace the absorption versus precipitation of calcium. Fluxes of acid-base relevant ions via the intestine and non-intestinal routes (gills, skin, kidney) were measured over 11 or 22 h periods during perfusion. All values are expressed as means  $\pm$  standard error.

## Results

Perfusion of the intestine with  $20 \text{ mM}$  (high  $\text{Ca}^{2+}$ ) instead of  $5 \text{ mM}$  calcium (controls) resulted in a 7-fold increase in the amount of precipitated carbonate and a 57% increase in the total intestinal bicarbonate secretion rate (sum of  $\text{HCO}_3^-$  equivalents in rectal fluid and precipitates). Perfusion with Hepes-buffered saline did not affect the overall bicarbonate excretion rate, but halved the proportion that was excreted as carbonate precipitates. There were no significant differences in the  $[\text{Ca}^{2+}]$  in rectal fluid of fish from the 3 treatments (means ranged from  $2.4\pm 0.4$  to  $3.6\pm 0.6 \text{ mM}$ ). Regardless of the perfusate  $[\text{Ca}^{2+}]$ , the % recovery of  $^{45}\text{Ca}$  in the rectal fluids was very high ( $\sim 80\%$ ) and virtually identical in all three treatments (Fig.1). The similar % recovery from the high  $\text{Ca}^{2+}$  saline was mainly due to large increase in precipitation of  $^{45}\text{Ca}$  as calcium carbonate (70%) rather than recovery in rectal fluid (Fig. 1). The osmolality of both rectal fluid and plasma was significantly reduced in fish perfused with the high  $\text{Ca}^{2+}$  saline, but net water absorption by the intestine was unaffected by any treatment. Blood pH and ammonia excretion via non-intestinal routes were also unaffected by perfusion with the different salines.

Fig. 1 : % of the  $^{45}\text{Ca}$  in the intestinal salines recovered in the rectal fluid and carbonate precipitates of flounder with intestines perfused over 72 h *in situ*.



### Conclusions

The large stimulation of total bicarbonate secretion by the intestine when calcium was elevated in the perfusion saline indicates that precipitation of calcium as carbonate may be a primary role of the bicarbonate secretion process. This is supported by acute *in vitro* experiments showing that bicarbonate secretion is stimulated very rapidly (within minutes) in response to elevation of calcium but not magnesium (see parallel presentation in this session by Grosell & Wilson). The reduced rectal fluid and plasma osmolality caused by high  $\text{Ca}^{2+}$  saline confirms the potential for an important role of bicarbonate secretion in osmoregulation. The up-regulation of bicarbonate secretion is very effective at precipitating at least 80% of the calcium entering the intestine, regardless of the initial concentration. Precipitation therefore also represents an important process in calcium homeostasis of marine teleosts by minimising absorption of imbibed calcium.

The stimulation of total intestinal bicarbonate secretion during perfusion with high calcium saline amounts to a simultaneous elevation of the net excretion of base via this route. This must have consequences for the acid-base balance of the whole animal. However, as no blood-acid-base disturbance was observed, whole animal acid-base balance must be maintained by the excretion of an equivalent

amount of excess acid via non-intestinal routes. The net excretion of  $\text{NH}_4^+$  via non-intestinal routes was not significantly altered, therefore an increase in the branchial excretion of other acidic equivalent ions (or decrease in excretion of basic equivalent ions) is likely responsible for maintaining acid-base homeostasis.

### **Acknowledgements**

The excellent technical assistance of Hanne Schaltz and Thomas Sørensen is greatly appreciated. R.W. Wilson was supported by BBSRC and Royal Society grants. M.Grosell was supported by the Danish Natural Research Council (grant no. 21-01-0255).

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## MECHANISMS OF INTESTINAL BICARBONATE SECRETION IN MARINE TELEOST FISH

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### EXTENDED ABSTRACT ONLY - DO NOT CITE

Bicarbonate is in many cases the dominant anion in the intestinal lumen of marine teleost fish and exceeds 100 mM in certain species (Fig. 1. and Walsh et al., 1991).

Considering the low extracellular bicarbonate concentrations in fish, such concentrations are in excess of electrochemical equilibrium across the intestinal epithelium (Grosell et al., 2001). This means that (secondary) active bicarbonate secretion must occur across the intestinal epithelium. Bicarbonate concentrations only increases slightly along the length of the intestine but in species investigated so far, all segments of the intestine can secrete bicarbonate at high rates.

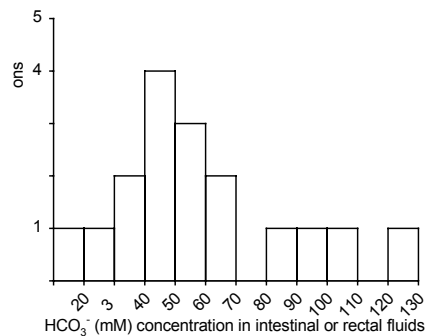


Figure 1. Frequency of 17 reported HCO<sub>3</sub><sup>-</sup> concentrations from intestinal or rectal fluids from 14 marine teleost fish

The substantial intestinal bicarbonate secretion does not seem to play a role in digestion since most or all reported studies were performed on un-fed animals. High intestinal bicarbonate concentrations are present in euryhaline species residing in seawater but virtually absent when in freshwater,

suggesting that bicarbonate secretion is involved in osmoregulation (Wilson, 1999).

Intestinal bicarbonate secretion occurs in exchange with chloride absorption via an apical chloride/bicarbonate exchanger (Grosell & Jensen, 1999; Grosell et al., 2001) and contributes significantly to overall chloride (and thereby water) absorption by the intestine. Carbonic anhydrase is fueling at least part of the bicarbonate secretion through hydration of CO<sub>2</sub> (Wilson et al., 1996). Ongoing studies employing pH-stat titrations have demonstrated that CO<sub>2</sub> arising from epithelial metabolism is the main source of the secreted bicarbonate in the European flounder. The protons arising from the CO<sub>2</sub> hydration must be extruded by the epithelial cells to prevent reversal of this carbonic anhydrase mediated reaction and thereby sustain sufficiently high bicarbonate concentrations for the chloride/bicarbonate exchange. The carrier(s) involved in the proton extrusion is the subject of current studies but it is clear that the extrusion must occur across the basolateral membrane to explain the substantial net base secretion by this epithelium. A basolateral proton pump could be driving this proton extrusion (Grosell et al., 2001) but basolateral sodium/proton exchange is another possible mechanism. The latter exchange process would be possible due to the sodium gradient across the basolateral membrane of the intestinal epithelial cells. This sodium gradient is established by the basolateral sodium-potassium ATPase which thus indirectly could be fueling intestinal bicarbonate secretion. Bicarbonate secretion appears to depend on serosal sodium (Grosell et al., 2001) which support the proposed involvement of a basolateral sodium/proton exchange mechanism. The possible involvement of a basolateral sodium:bicarbonate co-transport system seems unlikely since current studies revealed substantial bicarbonate secretion even in the absence of serosal bicarbonate.

Ongoing studies on the European flounder have demonstrated that reduced luminal pH and elevated luminal calcium concentrations both stimulate intestinal bicarbonate secretion. The result of the substantial bicarbonate secretion is a highly alkaline environment (Walsh et al., 1991; Wilson, 1999; Grosell et al., 2001) which facilitate calcium-carbonate precipitation. The functional significance of this precipitation is discussed in a parallel presentation (Wilson & Grosell).

#### **Acknowledgments**

The excellent technical assistance of Hanne Schaltz and Thomas Sørensen is greatly appreciated. M.Grosell is supported by the Danish Natural Research Council (grant no. 21-01-0255).

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**A NOVEL RENAL CHLORIDE CHANNEL, OmCIC-K,  
PLAYS AN IMPORTANT ROLE FOR FRESHWATER  
ADAPTATION IN MOZAMBIQUE TILAPIA**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

The kidney plays an important role in osmoregulation in freshwater teleosts, which are exposed to the danger of osmotic loss of Na<sup>+</sup> and Cl<sup>-</sup>. Since the primary function of the kidney in fresh water is to excrete excess water, it generates hypotonic urine by reabsorbing most of the filtered solutes. Nishimura et al. (1983) isolated and perfused nephron tubules from freshwater rainbow trout, *Oncorhynchus mykiss*, and showed that the net water movement across the distal tubule was very low, but the net Cl<sup>-</sup> reabsorption exists. These results suggest that in freshwater trout, which lack the loop of Henle, the distal tubule acts as a diluting segment such as the early distal segment of the frog kidney and the mammalian thick ascending limb of Henle's loop (TAL) (Burg and Green, 1973). The TAL of the mammalian kidney is involved in the urinary dilution and concentration processes by actively reabsorbing NaCl through the complex mechanism. Previous electrophysiological studies have demonstrated that two Cl<sup>-</sup> are taken up with one Na<sup>+</sup> and one K<sup>+</sup> by the TAL cells via the bumetanide-sensitive Na-K-2Cl cotransporter and then transported across the basolateral membrane. Major advances have been made over the past few years in our understanding of NaCl transport mechanisms in this nephron segment. This has been fueled by the molecular identification of the Na-K-2Cl cotransporter (NKCC2) (Gamba et al., 1994), K<sup>+</sup> secretory channels (ROMK) (Ho et al., 1993) and basolateral Cl<sup>-</sup> channel (CIC-K2) (Adachi et al., 1994; Kobayashi et al., 2001) expressed in the TAL. Despite abundant data in mammals, however, the identification of the ion transporters in the teleostean nephron was not done at all.

In the present study, to identify the molecular mechanisms of the teleost kidney, we have cloned OmCIC-K chloride channel from Mozambique

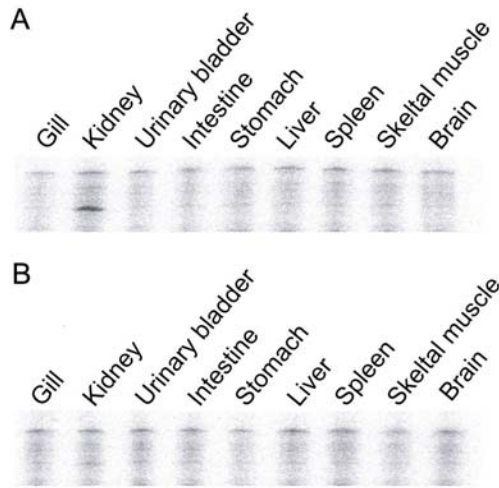


Figure 1. Tissue distribution of OmCIC-K in freshwater- (A) and seawater-adapted (B) tilapia by RNase protection assay.

tilapia, *Oreochromis mossambicus*, which is a homologue of the mammalian kidney-specific chloride channel, CIC-K. The cDNA of OmCIC-K encodes a protein, whose amino acid sequence has high homology to *Xenopus* and mammalian CIC-K (*Xenopus* CIC-K, 41.8%; rat CIC-K2, 40.9%; rat CIC-K1, 40.1%). In order to assess the role of OmCIC-K in the osmoregulation of tilapia, changes in expression of its mRNA was examined in various tissues after altering environmental salinities using the ribonuclease protection assay. The mRNA of OmCIC-K was expressed exclusively in the kidney and the expression level of mRNA was significantly increased in freshwater-adapted fish (Figure 1).

To investigate the physiological role of the OmCIC-K, we sought to determine its exact localization in the kidney of freshwater tilapia by immunohistochemistry. The immunoreactive cell was located in specific nephron tubules. In adjacent sections, immunoreaction of Na<sup>+</sup>, K<sup>+</sup>-ATPase was observed on the same tubules that were immunoreactive to OmCIC-K (Figure 2).

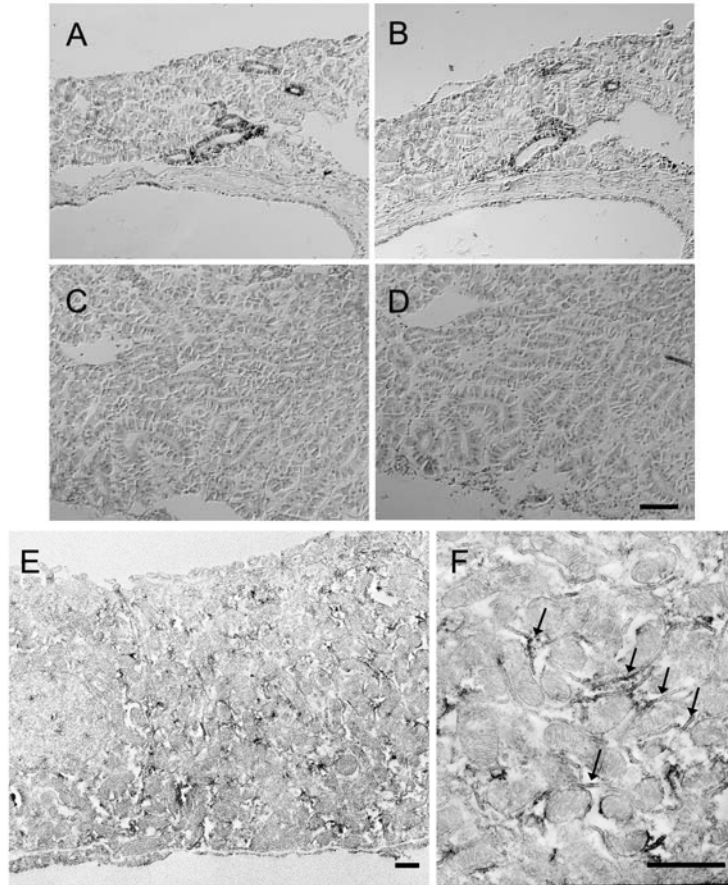


Figure 2. The serial sections of the kidney, stained with  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase antibody (A, C) and OmCIC-K antibody (B, D), in freshwater- (A, B) and seawater-adapted tilapia (C, D). Scale bar = 100  $\mu\text{m}$ .

E. Immunoelectron micrographs of the distal tubule stained with anti-OmCIC-K in tilapia kidney adapted to freshwater. F. a high magnification of E. Immunostaining (arrows) is mainly located on the infolding basolateral membrane. Scale bar = 500 nm.

To identify the OmCIC-K immunoreactive nephron, we performed the immunoelectron microscopy. Ultrastructural observations revealed that OmCIC-K-immunoreactive cells of freshwater-adapted fish had a few microvilli, a rich population of mitochondria and an extensive labyrinth of basolateral membrane infolding (Figure 2). Immunoreaction of OmCIC-K was recognizable on the structure of basolateral membrane infolding.

Therefore, the OmClC-K-positive tubules may correspond to the distal tubules of freshwater tilapia. Judging from the results obtained from mammalian ClC-K, OmClC-K is involved in the transepithelial Cl<sup>-</sup> reabsorption by the kidney of hyperosmoregulating, freshwater fish.

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**NaCl AND ACID/BASE REGULATORY MECHANISMS IN THE GILLS  
OF A EURYHALINE ELASMOBRANCH (*Dasyatis sabina*)**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

**Introduction**

The cellular mechanisms associated with NaCl and acid/base regulation in gills of elasmobranch fishes have not been well established, compared to those of teleost fishes (reviewed by Evans et al. 1999). Moreover, the influence of environmental salinity on the expression of these mechanisms in elasmobranch gills is unknown. The goals of this study are to 1) determine what mechanisms are present in the gill epithelium of a euryhaline elasmobranch (Atlantic stingray, *Dasyatis sabina*); and 2) determine if environmental salinity influences expression of these mechanisms. The specific ion transporters examined in this study are Na<sup>+</sup>,K<sup>+</sup>-ATPase, vacuolar proton-ATPase (V-H-ATPase), and pendrin.

In teleosts, Na<sup>+</sup>,K<sup>+</sup>-ATPase is localized to the basolateral membrane of mitochondrion-rich chloride cells, where it indirectly energizes NaCl secretion by the seawater teleost gill (reviewed by Evans et al. 1999). This transporter is also present in chloride cells of freshwater teleosts where it is presumably involved with active ion uptake (see Perry 1997). In seawater elasmobranchs, Na<sup>+</sup>,K<sup>+</sup>-ATPase-rich cells have been detected in the gills (Conley and Mallatt 1988), but the influence of salinity on Na<sup>+</sup>,K<sup>+</sup>-ATPase expression and function of the Na<sup>+</sup>,K<sup>+</sup>-ATPase-rich cells are unknown.

In teleosts, V-H-ATPase has been most extensively studied in gills of freshwater teleosts, where it is found on the apical membrane of chloride and pavement cells (Wilson et al. 2000). This transporter is hypothesized to actively excrete  $H^+$  and establish a favorable gradient for  $Na^+$  entry from freshwater (reviewed by Evans et al. 1999). In elasmobranch gills, V-H-ATPase has been detected in mitochondrion-rich cells of a seawater species (Wilson et al. 1997), but the influence of salinity on V-H-ATPase expression and function of the V-H-ATPase-rich cells are not known.

Pendrin is a newly described anion exchanger in mammals and has not been investigated in fishes or any other lower vertebrate. This exchanger functions as an apical  $Cl^-/HCO_3^-$  exchanger in V-H-ATPase-rich, bicarbonate-secreting intercalated cells in collecting ducts of the mammalian nephron (Royaux et al. 2001).

### **Materials and Methods**

Ten Atlantic stingrays were captured from freshwater lakes of the St. Johns River, Florida, USA. Five individuals were held captive in fresh water (freshwater stingrays), while the other five were acclimated to 32 parts per thousand (ppt) seawater for 1 week (seawater-acclimated stingrays). In addition, a group of 5 marine Atlantic stingrays was captured from Cedar Key, Florida, USA, and held captive in 32 ppt seawater (seawater stingrays).

From each animal, gill filaments were snap frozen in liquid nitrogen for immunoblot analysis and were fixed in 10 mmol/L phosphate buffered saline (pH 7.3) containing 3% paraformaldehyde, 0.05% glutaraldehyde, and 0.05% picric acid for immunohistochemical analysis.

For immunoblots, proteins from gill homogenates were separated by molecular weight using gel electrophoresis. The proteins were transferred to PVDF membranes that were incubated with the following antibodies: 1) a monoclonal to the  $\alpha$ -subunit of avian  $Na^+,K^+$ -ATPase; 2) a polyclonal to the B-subunit of insect V-H-ATPase, and 3) a polyclonal to human pendrin. Binding of antibodies to the PVDF was detected with an ECL detection kit.

For immunohistochemistry, sections of paraffin-embedded gill tissue were incubated singly with the above antibodies. Binding of antibodies was detected with an immunoperoxidase detection kit using DAB (brown stain) as the substrate. A double-labeling immunohistochemical procedure was also used to

detect binding of 2 antibodies on a tissue section, in which case DAB and another substrate (Vector SG; blue stain) were used to detect the first and second applied antibodies, respectively.

### **Results and Discussion**

Immunoblotting detected  $\text{Na}^+, \text{K}^+$ -ATPase, V-H-ATPase, and pendrin immunoreactivity in gills of freshwater, seawater-acclimated, and seawater stingrays. Densitometry analysis of the immunopositive bands revealed that relative abundance of the transporters was greatest in gills of freshwater stingrays, compared to gills of seawater-acclimated and seawater stingrays.

Immunohistochemistry detected  $\text{Na}^+, \text{K}^+$ -ATPase-rich, V-H-ATPase-rich, and pendrin positive cells in gills of freshwater, seawater-acclimated, and seawater stingrays. Immunostaining for all three transporters occurred in cells of gill lamellae and interlamellar regions in freshwater stingrays, but was primarily found in cells of interlamellar regions in seawater-acclimated and seawater stingrays. Regardless of salinity,  $\text{Na}^+, \text{K}^+$ -ATPase and V-H-ATPase immunoreactivity occurred in separate mitochondrion-rich cell types and were localized to the basolateral region of their respective cells. Pendrin immunoreactivity colocalized to the apical region of V-H-ATPase-rich cells.

In conclusion, results from this study provide immunochemical evidence for 1) the presence of  $\text{Na}^+, \text{K}^+$ -ATPase, V-H-ATPase, and pendrin in gills of the Atlantic stingray; and 2) an influence of salinity on expression of these transporters. The pattern of immunostaining for V-H-ATPase and pendrin is remarkably similar to bicarbonate-secreting cells of the mammalian nephron (Royaux et al. 2001), and we suggest that these cells are sites of apical  $\text{Cl}^- / \text{HCO}_3^-$  exchange in the stingray gill. In contrast, we propose that  $\text{Na}^+, \text{K}^+$ -ATPase-rich cells are sites of apical  $\text{Na}^+ / \text{H}^+$  exchange through a currently unidentified transporter(s).

### **Acknowledgements**

We thank Keith Choe, Sarjeet Gill, William Harvey, Ines Royaux, and Jill Verlander Reed for their assistance. Research was funded by EPA-STAR Graduate Research Fellowship U-915419-01-0 (PMP) and NSF Grant IBN-0089943 (DHE).

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**SALINITY ACCLIMATION IN RAINBOW TROUT:  
MOLECULAR AND PHYSIOLOGICAL RESPONSES**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Salinity acclimation occurs in a limited number of teleost species. The rainbow trout, while having a restricted capacity to tolerate abrupt transfer from freshwater to full seawater, is capable of acclimating to gradual increases in salinity. The response to gradual salinity increases in the trout has not been fully characterized at the molecular level. Evidence for the involvement of a number of important gill transport proteins, channels and receptors during acute seawater exposure has been demonstrated in several teleost species including: Brown trout (Madsen et al. 1995), the Atlantic salmon (Singer et al. 2002) and the euryhaline killifish (Singer et al. 1998). To better characterize the acclimatory responses, we have examined changes in mRNA levels and protein content of salinity-responsive genes from the gill in rainbow trout.

Juvenile trout were gradually acclimated to increased salinity over 5 days (Day 1: 33% full seawater; day 2: 50% full seawater; day 5: 66% full seawater). Plasma cortisol, glucose, Na<sup>+</sup> and Cl<sup>-</sup> levels and gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity were measured from each fish at each time point. The gill Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit, CFTR, and GR mRNA abundance were measured using quantitative Real-Time PCR.

Plasma  $\text{Na}^+$  and  $\text{Cl}^-$  levels were undisturbed at each salinity suggesting that the trout was capable of osmoregulation. Plasma cortisol levels also showed no change, while gill GR mRNA levels increased 3-fold over FW levels after 24h at 33% full seawater and peaked (4-fold increase) at day 3 at 50% seawater. The potential for increased GR capacity may be involved in the regulation of specific genes regulated mediated through glucocorticoid responsive elements (GREs). Trout gill CFTR mRNA levels increased with salinity exposure, suggesting a role for this chloride channel in trout salinity acclimatory response. The response is similar to both killifish CFTR and the Atlantic salmon CFTR I isoform which both show sustained increases in mRNA levels following abrupt exposure to full seawater (Singer et al. 1998, Singer et al. 2002). In contrast to CFTR, gill  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit mRNA levels rose transiently, peaking at 24 hours and 33% full seawater, followed by a significant decrease in mRNA levels compared with freshwater at both 3 day and 5 day (50% and 66% seawater). The reduced trout gill  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit mRNA levels at 3 and 5 days reflect the decline in gill  $\text{Na}^+, \text{K}^+$ -ATPase enzyme activities. Overall, this study demonstrates that salinity acclimation in rainbow trout is mediated by molecular pathways previously described in other teleosts.

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## Acknowledgements

This study was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) operating grant to MMV.

## NATRIURETIC PEPTIDE SYSTEM GOVERNS DIVERSE

### OSMOTIC ADAPTABILITY OF EELS

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### EXTENDED ABSTRACT ONLY – DO NOT CITE

#### The Natriuretic Peptide System in Eels

Three natriuretic peptides (atrial, ventricular, and C-type NP; ANP, VNP and CNP), and four NP receptors (NPR-A, -B, -C and -D) have been identified in eels, *Anguilla japonica* (Fig. 1). NPR-A and -B are guanylyl cyclase-coupled receptors that utilize cGMP as an intracellular messenger to exert

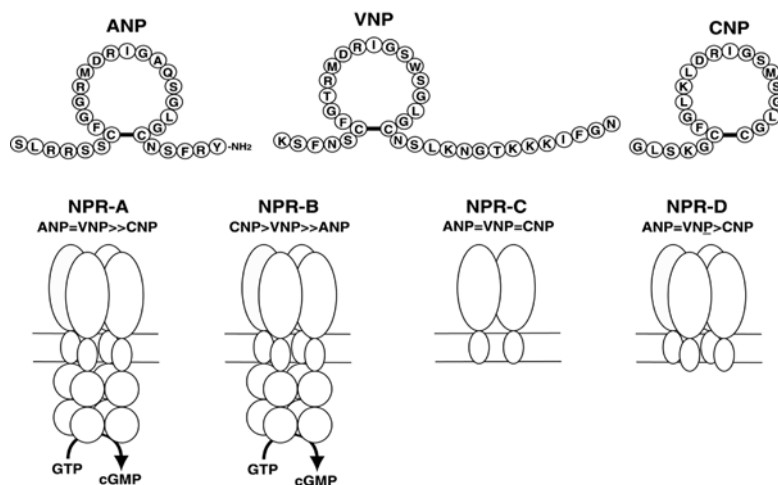


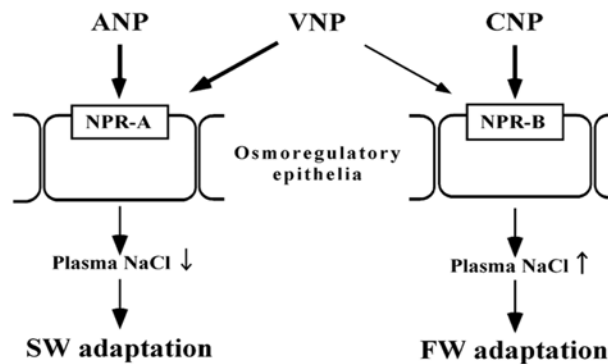
Figure 1. Natriuretic peptides and their receptors in eels.

biological actions. ANP and VNP have high affinity to NPR-A and CNP (and to a lesser extent, VNP) to NPR-B. The biological functions of NPR-C and NPR-D have not been fully elucidated. However, NPR-C may act as a buffer to regulate the peptide levels in plasma and at target tissues, since all three peptides bind NPR-C with equally high affinities and NPR-C is present in high densities in all tissues where NP effects have been demonstrated. NPR-D is expressed exclusively in the brain, thus indicating some biological function of guanylyl cyclase-deficient receptors

### ANP Is a Seawater (SW)-Adapting Hormone

ANP is a volume-regulating hormone in mammals that is secreted in response to hypervolemia and it decreases blood volume by stimulating the excretion of both water and NaCl (Fig. 2). In eels, however, ANP secretion is stimulated by hypernatremia and secreted ANP promotes SW adaptation by inhibiting the uptake and stimulating the extrusion of Na<sup>+</sup> ions specifically (Loretz and Pollina, 2001). The Na<sup>+</sup>-specific effect in eels is supported by the fact that ANP infusion at physiological doses decreases urine flow but increases urine Na<sup>+</sup> concentration in SW eels (Takei and Kaiya, 1998). Actually, ANP is released upon transfer of eels to SW and released ANP inhibits drinking of environmental SW and subsequent intestinal absorption of NaCl from ingested SW. In this way, ANP

Figure 2. Interaction of natriuretic peptides for adaptation to diverse salinity



ameliorates a sudden increase in plasma Na<sup>+</sup> concentration and facilitates the survival of initial phase of SW adaptation. After the initial role, ANP disappears from blood quickly and participates in long-term adaptation to SW by stimulating cortisol secretion. Cortisol is known to differentiate branchial chloride cells to a SW type (McCormick, 1994).

### **CNP Is a freshwater (FW)-Adapting Hormone**

CNP is a neuropeptide or a paracrine factor in peripheral tissues and is not involved in osmoregulation in mammals. In eels, however, CNP appears to be an important hormone for FW adaptation by stimulating the uptake of NaCl from media (Fig. 2). Supportive evidence is that considerable amounts of CNP is circulation in the plasma of FW eels, and that CNP-specific NPR-B is expressed abundantly in osmoregulatory organs of FW eels (Takei et al., 2001). In fact, infusion of CNP into FW eels increases plasma Na<sup>+</sup> concentration and increases <sup>22</sup>Na uptake from environmental water (J.C. Rankin and Y. Takei, unpublished data). This is opposite to the decrease of plasma Na<sup>+</sup> concentration observed after ANP infusion into SW eels (Fig. 2).

### **Natriuretic Peptide System and Euryhalinity**

Eels are euryhaline, migratory species that readily adapt to both FW and SW environments. Judging from the data mentioned above, ANP and CNP are important for eel's excellent ability to adapt to diverse salinity environments. VNP is secreted constitutively into the circulation and its plasma concentration is relatively constant. Therefore, VNP may supplement the action of ANP and CNP for adaptation to SW and FW, respectively, as VNP has high affinity to both NPR-A and NPR-B (Fig. 2). Taken together, the natriuretic peptide system appears to play pivotal roles in diverse osmotic adaptability (euryhalinity) of eels (Takei and Hirose, 2002). It is somewhat surprising that the peptides in the same family that share more than 60% sequence identity act to promote adaptation to opposite environmental salinity.

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### **Acknowledgements**

The author thanks many collaborators, particularly Dr. Shigehisa Hirose of Tokyo Institute of Technology, Dr. Akiyoshi Takahashi of Kitasato University for encouragement and discussion. He also thanks graduate students and postdocs of my laboratory who conducted most of the works presented here.

**PYLORIC CAECA IN CHINOOK SALMON:  
OSMOREGULATORY FUNCTION  
AND RESPONSIVENESS TO CORTISOL**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Pyloric caeca, found in some, but not all fish, are blind-end outpocketings of the anterior intestine. In salmon and trout, the caeca are numerous and account for a major proportion of the surface area of the post-gastric intestinal tract. Although the pyloric caeca are known to be a major site for nutrient absorption (Collie, 1985; Buddington and Diamond, 1987), little is known of their potential role in the intestine's other major function, salt and water balance. In the present study, we demonstrate that the pyloric caeca of chinook salmon (*Oncorhynchus tshawytscha*) have a functional role in osmoregulation, are a site for adaptive changes during seawater entry, and that these changes may be directly regulated by cortisol during parr-smolt transformation.

**Osmoregulatory changes during seawater adaptation**

We first established a technique to make volumetric measurements of fluid absorption on individually isolated caeca. We also measured concurrent changes in mucosal Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in these same caeca, since this enzyme is part of the mechanisms for epithelial ion transport. Fluid absorption was found

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to be highly dependent on  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity, as evidenced by a 92% inhibition of fluid absorption by ouabain ( $10^{-4}$  M). Adaptation of salmon to seawater for 10 days or 6 months resulted in significantly elevated rates of both fluid absorption and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity over salmon remaining in fresh water, indicating that the pyloric caeca undergo functional changes that are adaptive for seawater. Comparing the relative amounts of total fluid absorbed between the caeca and posterior intestine (a known osmoregulatory site) suggests that the caeca make a much more significant contribution to the absorption of imbibed water when the salmon are adapted to seawater.

### **Cortisol stimulates osmoregulatory mechanisms**

We next asked whether the seawater-adapting hormone, cortisol, could stimulate functional changes in the caeca associated with seawater adaptation, as has been shown for the posterior intestine (Veillette et al., 1995). Cortisol implanted into fresh water adapted salmon, at 50  $\mu\text{g/g}$  body weight for 10 days, significantly elevated plasma cortisol and stimulated both fluid absorption rate and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in the caeca to levels equal to those seen during seawater adaptation. Thus, the pyloric caeca are a site for adaptive, osmoregulatory changes in the salmon intestine that may be regulated by cortisol. Additionally, the concurrent increases in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and rate of fluid absorption, resulting from seawater adaptation or cortisol implants, suggest a functional link between the two during the development of osmoregulatory mechanisms in the caeca.

### **Parr-smolt transformation and responsiveness of $\text{Na}^+$ , $\text{K}^+$ -ATPase to cortisol**

Chinook salmon may migrate to sea during their first or second year of life in spring or summer. In our facility, they undergo parr-smolt transformation at these times. We examined seasonal changes in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity of the pyloric caeca and found that this enzyme increases during parr-smolt transformation in both yearlings and underyearlings. In these same fish, the *in vitro* responsiveness of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity to cortisol was assessed by exposing explants of caeca to cortisol for six days in culture. A response was restricted to the several months immediately preceding endogenous increases in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and a month afterward. At a time when explants were responsive to cortisol, *in vitro* exposure to 0.01-10  $\mu\text{g/ml}$  cortisol maintained  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity over controls (0  $\mu\text{g/ml}$  cortisol) in a dose-dependent manner. These results show that the pyloric caeca exhibit increased  $\text{Na}^+$ ,  $\text{K}^+$ -

ATPase activity that is preadaptive for seawater entry during parr-smolt transformation, and that changes in tissue responsiveness to cortisol may play a part in regulating these changes.

### **Conclusion**

The numerous pyloric caeca of salmonids, in addition to their role in nutrient uptake, appear to have a major role in osmoregulation. This is evidenced not only by its large surface area and absorptive capacity, but also by our demonstration of functional changes during seawater adaptation and parr-smolt transformation, and the response to cortisol. These findings suggest that the pyloric caeca deserve attention in future studies examining osmoregulatory changes in the intestine of salmon, and other euryhaline fish.

### **Acknowledgements**

We thank Dr. Ronald J. White (Eastern Washington University) for invaluable discussions and laboratory expertise. P.A.V. was supported by a Ph.D. Scholarship from the William Evans Fund (University of Otago) during these studies. This research was funded by Otago Research Grants to G.Y.

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**THE ENDOCRINE CONTROL OF  $\text{Na}^+, \text{K}^+$ -ATPASE  $\alpha$ -SUBUNIT  
EXPRESSION AND ENZYME ACTIVITY IN THE GILL OF ATLANTIC  
SALMON: A COMPARISON OF A NATURAL HYPOTHYROID  
MODEL WITH THYROID HORMONE TREATMENT**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Thyroid hormones have been implicated in many of the physiological changes that occur during salmon smoltification, however the role of thyroid hormones in osmoregulation has been suggested to be a modulatory one, interacting with growth hormone and cortisol to regulate the osmoregulatory machinery in the gill. Here we present data from two experiments seeking to elucidate the specific localization and functional actions by thyroid hormones during smoltification in the Atlantic salmon (*Salmo salar* L.).

The first experiment compared endocrine and branchial smoltification-related changes in an anadromous strain and a landlocked strain of Atlantic salmon. Previously, we have reported the osmoregulatory differences between these landlocked and anadromous strains and demonstrated a 3-fold difference in

Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and the inability for the landlocked strain to survive long term in seawater (Nilsen *et al.*, in press). We show now that in the landlocked strain circulating thyroxine (T<sub>4</sub>) and growth hormone (GH) peak levels were 50% those of the anadromous strain during smoltification. The hormone levels increased in the anadromous strain in April and both strains reached their peak levels in May. Moreover, the number of Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit immunoreactive cells and the intensity of the immunoreaction was higher in the anadromous compared with the landlocked strain.

In a second experiment, anadromous Atlantic salmon parr were given a treatment with L-thyroxine by immersion for 6 weeks, from early March to mid April. Thyroxine-treatment increased GH levels up to 4-fold those of controls, reaching peak values after 6 weeks of treatment, levels which were not obtained by controls until 5 weeks later. The treatment however, had no effect on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, which increased in both groups from week 3 until the end of the treatment. These results appear consistent with previous studies showing little or no effect of thyroid hormones on osmoregulation. However, further immunocytochemical analysis also revealed that there was an increased number of Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit immunoreactive cells in the thyroxine-treated group, especially in the secondary lamellae. The thyroxine treatment increased the number of chloride cells and the Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit within the cells.

In addition, *in situ* hybridization revealed expression of thyroid hormone receptor  $\beta$ -subunit (TR- $\beta$ ) in chloride cells, i.e. a target for thyroid hormone action on osmoregulatory capacity in the gill. These data combined suggest that thyroid hormones can regulate key osmoregulatory elements in the gill directly; and indirectly through the GH system. These data support a modulatory role of thyroid hormones in osmoregulation, acting on the proliferation of chloride cells and/or the expression of the Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit. The Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, on the other hand, is dependent on the availability of other critical factors, such as growth hormone, cortisol and their receptors, the Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\beta$ -subunit, and hitherto undefined variables.

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### **Acknowledgements**

The Research Council of Norway funded this work through the Strategic Research Programme 'Smolting in Atlantic salmon: the interrelationship between physiological, endocrine and immunological changes, and their genetic regulation' (136260).



**IONIC REGULATION IN SOFTWATER-ACCLIMATED RAINBOW  
TROUT: A ROLE FOR GLUCOCORTICOID AND  
MINERALOCORTICOID RECEPTORS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

The main corticosteroid hormone in fish, cortisol, plays a dual role in contributing to the regulation of both carbohydrate metabolism and salt/water balance. Until recently, both the mineralocorticoid and glucocorticoid actions of cortisol were considered to be mediated by a single corticoid receptor of the glucocorticoid receptor (GR) subtype (Mommensen et al., 1999). The cloning from rainbow trout testis of a novel steroid receptor that exhibited high homology to mammalian and amphibian mineralocorticoid receptor (MR) cDNA sequences at both nucleotide and amino acid levels, as well as steroid binding characteristics consistent with those of other MR, strongly suggests the presence in trout of a mineralocorticoid-like receptor (rtMR) (Colombe et al., 2000). Whereas GR have been characterised in a number of fish species and tissues, the tissue distribution, physiological function and regulation of MR in fish are not yet clear.

In the present study, the acclimation of rainbow trout to ion-poor water has been used as a tool to investigate the function and regulation of GR and MR in the freshwater fish gill. When acclimated to soft water, rainbow trout experience a proliferation of the ion-transporting chloride cells of the branchial epithelium, which contributes to the maintenance of ionic homeostasis by enhancing

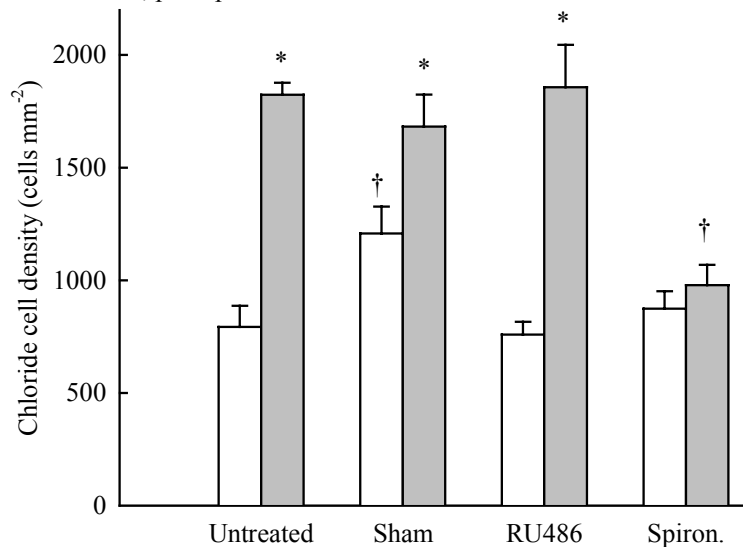
branchial ion uptake. Chloride cell proliferation in softwater-acclimated trout is thought to be stimulated by cortisol, first, because several studies have reported transient elevations of circulating cortisol concentrations during exposure to ion-deficient water (e.g. Perry and Laurent, 1989), and second, because cortisol treatment (in the absence of an ionoregulatory challenge) has been found to induce chloride cell proliferation (e.g. Bindon et al., 1994).

Rainbow trout (*Oncorhynchus mykiss*) were either maintained in dechlorinated city-of-Ottawa tap water (tap water; in mmol L<sup>-1</sup>, [Na<sup>+</sup>] = 0.163, [Ca<sup>2+</sup>] = 0.397, [K<sup>+</sup>] = 0.021 and [Cl<sup>-</sup>] = 0.173) or were provided with dechlorinated tap water diluted with reverse osmosis water (artificial soft water; in mmol L<sup>-1</sup>, [Na<sup>+</sup>] = 0.032, [Ca<sup>2+</sup>] = 0.096, [K<sup>+</sup>] = 0.007 and [Cl<sup>-</sup>] = 0.050). Fish were exposed to the softwater condition by a gradual increase in the proportion of RO water over a 24 h period, and exposure periods ranged from 24 h to 7 days. Trout in each acclimation condition were allocated to one of four treatment groups; untreated (control), a single intraperitoneal injection of warm coconut oil (sham; 0.005 mL g<sup>-1</sup> body mass), coconut oil containing the GR antagonist RU486 (RU486; 0.5 mg g<sup>-1</sup> body mass), or coconut oil containing the MR antagonist spironolactone (spironolactone, 0.1 mg g<sup>-1</sup> body mass). Plasma cortisol and ion concentrations, and branchial chloride cell proliferation, Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, GR protein expression, and GR and MR mRNA abundance were examined as a function of acclimation condition and/or treatment.

Softwater acclimation elicited chloride cell proliferation, a response that was blocked by treatment with the MR antagonist spironolactone, but not by the GR antagonist RU486 (Fig. 1). This finding provides further evidence for the presence of a mineralocorticoid-like receptor in rainbow trout, as proposed by Colombe et al. (2000), and implicates MR rather than GR in eliciting the response to the ionoregulatory challenge of softwater exposure. Indeed, branchial GR protein content decreased significantly as a result of acclimation to ion-poor water. Chloride cell proliferation in softwater-acclimated fish occurred in the absence of any significant elevation of plasma cortisol levels, or significant change in branchial Na<sup>+</sup>-K<sup>+</sup>-ATPase activity. Plasma ion levels were unaffected by softwater acclimation, implying that the physiological responses to this ionoregulatory challenge were sufficient to maintain ionic homeostasis.

In conclusion, the results of the present study support the hypothesis that rainbow trout possess both GR and MR, and that these receptors may be differentially regulated. Further, the data confirm that cortisol plays an

important role in mediating the physiological responses to softwater acclimation in rainbow trout, perhaps via MR.



**Figure 1.** The effect of acclimation condition and treatment on chloride cell density. Values are means  $\pm$  SEM ( $N = 4$ ). \* indicates a significant difference between tap water and softwater-acclimated fish within the same treatment group; † indicates a significant difference within an acclimation condition from the untreated group (two-way ANOVA,  $P < 0.05$ ). Modified from Sloman et al. (2001).

### Acknowledgements

Thanks are extended to Dr. K. Sloman and R. Sathiyaa. This study was funded by Natural Sciences and Engineering Research Council (NSERC) of Canada research and equipment grants, as well as Canada Foundation for Innovation and Ontario Innovation Trust infrastructure grants to KMG and to MMV.

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**ENDOCRINE PATHWAYS FOR THE DISRUPTION OF THE  
PARR-SMOLT TRANSFORMATION BY ESTRADIOL AND  
NONYLPHENOL IN ATLANTIC SALMON.**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

The parr-smolt transformation is a hormone-driven developmental process that is adaptive for downstream migration and seawater entry. Recently, environmental estrogens such as nonylphenol have been implicated in reduced returns of Atlantic salmon in the wild (Fairchild *et al.* 1999). Previous research has shown that sex steroids interfere downstream migratory behavior and the development of salinity tolerance that normally occurs during smolting (Madsen *et al.*, 1997; Munakata *et al.* 2000). The present study was designed to determine the endocrine pathways through which sex steroids and their agonists may act to affect the parr-smolt transformation.

Junvenile Atlantic salmon were injected with 0.5, 2, 10, 40 and 150 ug/g branched 4-nonylphenol and 2 ug/g estradiol-17 $\beta$  during the parr-smolt transformation in late March and sampled 1 and 2 weeks after first exposure. Treatment with estradiol and 150 ug/g nonylphenol resulted in lower salinity tolerance after two weeks of exposure. Plasma insulin-like growth factor I (IGF-I) was 30-60% lower in estradiol and 150 ug/g nonylphenol treated fish after 1 and 2 weeks of treatment. Plasma growth hormone was elevated at intermediate doses of nonylphenol, but there was no effect of higher doses of

nonylphenol or estradiol. Plasma cortisol was not affected by nonylphenol or estradiol. Plasma thyroxine showed a strong dose dependent decrease in response to nonylphenol and estradiol after one week, but no effect after two weeks. Plasma triiodothyronine was 25-60% lower in estradiol and 150 ug/g nonylphenol treated fish after 1 and 2 weeks of treatment

The results indicate that plasma IGF-I is the likely endocrine pathway for the effects of estrogenic compounds on osmoregulation during smolting. The thyroid axis is also sensitive to estrogenic compounds, and plasma thyroxine is negatively affected by nonylphenol at relatively low doses. Thus, thyroid-dependent processes during smolting, such as imprinting and migration, may also be impacted by estrogenic compounds.

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**PLASMA CHLORIDE CONCENTRATIONS ARE NOT A GOOD  
MEASURE OF OSMOREGULATORY OR ACID-BASE STATUS IN  
EELS (OR OTHER FISH?)**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

**Introduction**

Plasma chloride ion concentration is easy to measure and is often used as an indication of osmoregulatory status, on the assumption that it is proportional to total ionic concentration. The plasma sodium minus chloride concentration difference is often used as a rough approximation to strong ion difference (SID). Since changes in SID are largely due to changes in bicarbonate ion concentration, it is used as an indicator of acid-base status. In the European eel, *Anguilla anguilla*, the ratio of plasma sodium to chloride concentrations is variable (Kirsch, 1972). There is an inverse relationship between plasma chloride and bicarbonate concentrations but this is nowhere near enough to account for the variation in sodium to chloride concentration ratio found (Farrell and Lutz, 1975). As part of a study on transfer to sea water of juvenile eels used in the Danish stocking programme, complete analyses of plasma inorganic ion concentrations were carried out with surprising results.

**Materials and Methods**

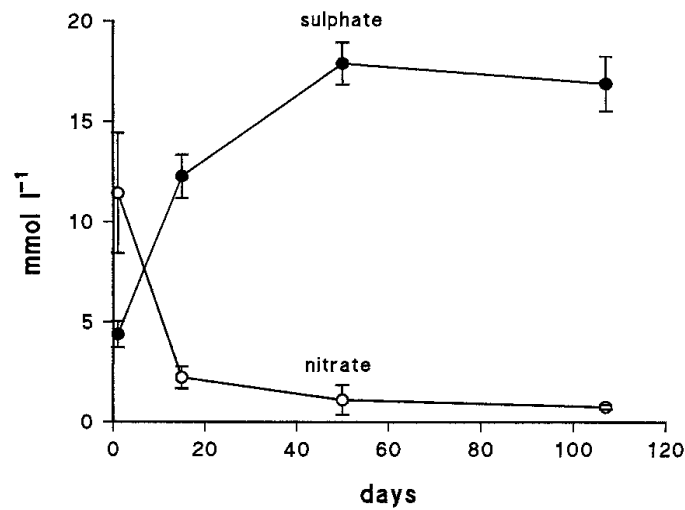
Eels were obtained from an eel farm in Jutland where they had been intensively reared in heated, recirculated, oxygenated freshwater. They were acclimated to running freshwater (Kerteminde tapwater) at 15°C. Blood samples were taken from the caudal vessels of eels maintained in freshwater or following

acclimation to 28 ppt sea water, and the plasma analysed using a Dionex 4500i ion chromatograph. The freshwater group was found to have very high sulphate concentrations. A fresh batch was obtained from the eel farm and groups sampled the following day and at intervals thereafter.

### Results and Discussion

One day after collection the eels were found to have very high plasma nitrate levels. Nitrite concentrations were extremely low or undetectable. Presumably ammonia produced by the eels in the intensive recirculatory rearing system had been converted to nitrite and then nitrate by biological filters. Nitrate concentrations decreased over a period of days, mirrored by an increase in sulphate concentrations, which attained around 35mequiv. l<sup>-1</sup> after 50 days (fig. 1)

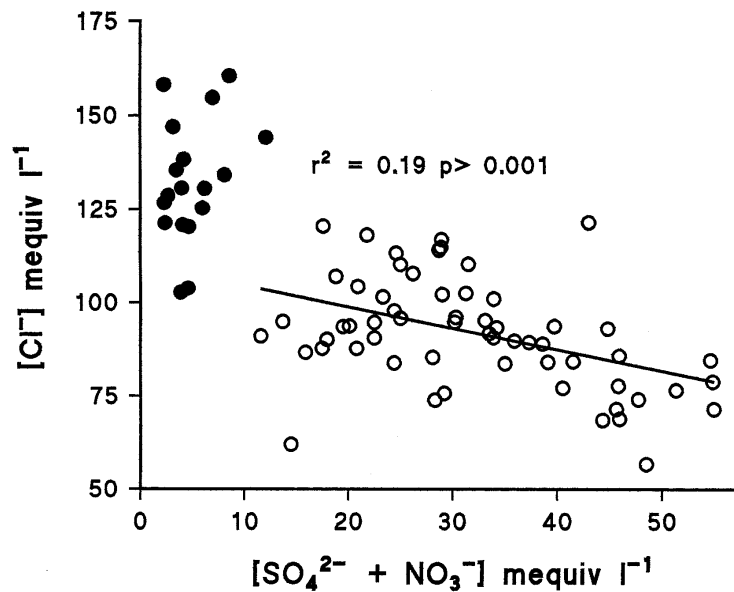
Figure 1. plasma nitrate and sulphate concentrations.



There was a significant negative correlation between plasma chloride and sulphate concentrations in freshwater eels ( $r^2 = 0.1$ ,  $p < 0.05$ ). There was an even better correlation between sulphate + nitrate and chloride concentrations ( $r^2 =$

0,19,  $p < 0.001$ ; fig. 2). Following transfer to sea water, plasma chloride concentrations rose and sulphate concentrations fell rapidly. There was no correlation between plasma chloride and sulphate or sulphate + nitrate concentrations in plasma from sea water eels (solid circles, fig. 2)

**Figure 2. Relationship between plasma chloride and sulphate + nitrate concentrations.**



Plasma ion levels in a population of wild eels from the rivers Loire and Nive in France were also measured. In freshwater plasma chloride was  $58.4 \pm 16.5$  and sulphate  $19.1 \pm 10.2$   $\text{mmol l}^{-1}$  (means  $\pm$  standard deviations to illustrate variability,  $n = 32$ ). The highest sulphate concentration found was  $40.3$   $\text{mmol l}^{-1}$  ( $80.6$   $\text{mequiv l}^{-1}$ ). Plasma nitrate concentrations were low ( $< 1$   $\text{mmol l}^{-1}$ ) but inorganic phosphate was high ( $8.6 \pm 3.7$   $\text{mmol l}^{-1}$ ). A group of these fish was transferred to sea water and after 1 month the following plasma concentrations were found: chloride  $142.7 \pm 16.5$ ; sulphate  $7.8 \pm 1.6$ ; inorganic phosphate  $4.0 \pm 2.2$   $\text{mmol l}^{-1}$  (means  $\pm$  SDs,  $n = 13$ ).

The eels in the present study were all starved. A number of studies (e.g. Goss and Perry 1994) have shown no measurable uptake of chloride across the gills of the American eel, *Anguilla rostrata*. Presumably the slow losses of chloride across the gills and in the urine are normally balanced by dietary uptake. The present study shows that chloride can be partially replaced by other ions, including sulphate, nitrate and inorganic phosphate. How these enter the blood from the water or from tissue breakdown is unknown.

Plasma chloride concentration is obviously useless as an indicator of osmoregulatory or acid-base status in the eel. Could the same apply to other fish? In freshwater-acclimated American shad, *Alosa sapidissima*, plasma chloride concentration at winter (non-feeding) temperatures fell to 38mM compared to 93mM in fish maintained at 24°C (Zydlewski and McCormick, 1997). Since osmolality only fell from 318 to 288 mOsm kg<sup>-1</sup> something must presumably have been replacing chloride. In rainbow trout over a third of the plasma chloride was replaced by bromide after 2 weeks in freshwater (1.39mM chloride) with 1mM bromide added (Stormer et al, 1996). Teleosts seem to be able to substitute plasma chloride with other ions to a considerable degree without apparent ill effects. This should be borne in mind when interpreting measurements of plasma chloride concentrations.

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#### **Acknowledgements**

This work was supported by the Elisabeth og Knud Petersens Fond and European Union grant Q5RS-2001-018369.



**ORIGINS AND CONSEQUENCES OF MITOCHONDRIAL  
DECLINE IN NUCLEATED ERYTHROCYTES**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

Erythrocytes have many roles in ion regulation and respiratory gas exchange, processes that depend upon energy provided by mitochondrial metabolism. Within a few months after red cells are released into the circulation, they exhibit changes in structure and function. Aging is accompanied by reduced membrane fluidity, losses in organelles and a reduction in gene expression and protein synthesis. The impact of cellular aging on the responsibilities of erythrocytes has only recently been studied. We have previously shown that erythrocytes defend ion and gas exchange properties as they age (1). Conversely, aging has a profound effect on the ability of cells to mount heat shock responses (2). Recent work has focused on the impact of age-dependent changes in mitochondria and bioenergetics. Aging erythrocytes experience dramatic losses in mitochondria. These changes could reduce the capacity of the older erythrocytes to perform energy dependent functions. Age-induced changes in mitochondria could also increase the vulnerability of aged cells to mitochondria-initiated programmed cell death, or apoptosis.

We mounted a series of studies to assess the origins and consequences of mitochondrial losses (3). What mechanisms are responsible for causing the changes in bioenergetics during aging? Do these mitochondrial changes alter energetics? Do the changes increase the propensity for apoptosis? In addition to investigating the effects of aging on erythrocyte energetics (discussed below), we assessed the relationship between cell aging periodicity and seasonal remodelling of tissue energetics. It is well known that Winter acclimation causes significant changes in muscle bioenergetics (4). Because fish experience a round of erythropoiesis in Spring, they enter Winter with aged cells. We examined the hypothesis that muscle remodelling was related to the age of the erythrocyte

population. Although older cells retain their capacity for anti-oxidant protection (3), it is possible that the combination of cold and cell age alters the rigidity of erythrocyte membranes, altering oxygen delivery/antioxidant protection. Specifically, we tested the hypothesis that seasonal changes in fish muscle energetics were imposed through these effects on the ability of erythrocyte to penetrate the vasculature.

Trout erythrocytes were separated into age classes using inherent differences in buoyant density. Based upon enzyme activities, the oldest cells demonstrate had lost about half of their mitochondria. Studies with isolated mitochondria showed there were no changes in mitochondrial enzymes stoichiometries, effects that are often seen in models of organismal aging. The oldest cells showed a profound decline in mtDNA transcripts, due to reductions in both transcription and mtDNA copy number but we saw no evidence for qualitative changes mitochondrial gene expression. Collectively, these data suggest that mitochondrial losses arise through controlled reductions in synthesis of mitochondrial precursors, rather than an accumulation of spontaneous defects.

Age also appeared to have pronounced effects on energy metabolism. Older cells had lower basal respiration than young cells. Despite the reduced rates of respiration, there was no effect of age on tissue ATP levels. In addition, the maximal respiratory rates were similar in old and young cells. Thus, old cells retain a capacity for elevating respiration, even if basal respiratory rates are lower than in young cells. The reduction in metabolic rate probably reflects the lower costs of other metabolic processes.

Mammalian erythrocytes lack nuclei and are unable to induce apoptosis. It was not known if nucleated erythrocytes of fish retain the capacity to induce apoptosis. Apoptosis could not be induced in either whole blood, young or old erythrocytes by pro-oxidants, mitochondrial inhibitors or the mitochondrial permeability transition agonist staurosporine. Surprisingly, treatment with cyclosporin A caused caspase 3 activation, DNA laddering and LDH leakage, but only in young cells. Fluorescence microscopy, caspase inhibitor studies suggest that mitochondria are not involved in erythrocyte apoptosis. However, the ability of erythrocytes to undergo apoptosis has important ramifications to the use of erythrocytes as biomarkers.

Collectively, these studies suggest that the mitochondrial changes with aging do not compromise cellular function, although trout erythrocytes can initiate apoptosis by non-mitochondrial pathways.

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**EFFECTS OF EXOGENOUS CORTISOL ON THE EXPRESSION OF  
CORTISOL AND NATRIURETIC PEPTIDE B RECEPTORS MRNA IN  
GILL EPITHELIA OF JAPANESE EELS, *ANGUILLA JAPONICA*.**

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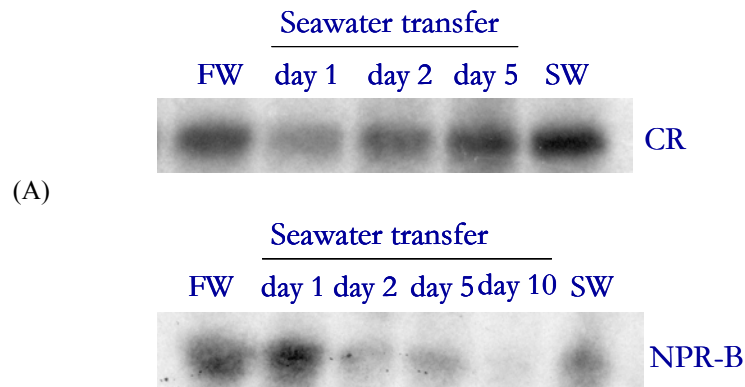
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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Abstract**

Cortisol, a major corticosteroid factor secreted by the interrenal gland, has been proven to be important in hypo-osmoregulation in teleost fish (Mayer et al., 1967). In the initial phase of seawater acclimation, a marked transitory increase in plasma cortisol levels was detected. The duration of the elevated plasma cortisol level was correlated with morphological changes in the gill epithelium, whereby a drastic cellular and functional reorganization occurred to adjust to this short-term osmotic crisis. Considerable numbers of studies have demonstrated that exogenous cortisol treatment stimulated Na<sup>+</sup>/K<sup>+</sup>-ATPase activities, mitochondria content and cellular differentiation of both freshwater and seawater CC. Nevertheless, the extent of cell proliferation and differentiation as well as the biochemical changes in cortisol treated freshwater fish were significantly lower than that of seawater fish (Wong and Chan, 2001). This could be explained as seawater acclimation is controlled by a complex endocrine regulatory mechanism in mediating the whole course of adaptation. Therefore information on the interactions of different seawater adapting hormones during seawater acclimation is essential. In the first part of present study, we conducted a molecular study to determine the effects of exogenous cortisol treatment on the expression of cortisol receptor (CR) and natriuretic peptide B receptor (NPR-B) in gill epithelia of freshwater adapted eels. The effects of 2-day intramuscular injection of 2 µg/g of cortisol were compared with the seawater-adapted fish. The fish were scarified on day 1, 2, 5 and 10. For CR expression in seawater acclimating fish (fig. 1A), a transient reduction of gill CR followed by a progressive increase to the level comparable to freshwater condition. Exogenous

cortisol treatment, however, stimulated a transient increase of CR transcript, followed by a downregulation of the receptor. The results indicated that a transient increase in plasma cortisol level arising from exogenous injection could not maintain a steady level of CR expression in gill epithelia as compared to the seawater acclimating fish. For NPR-B (fig. 1B), an initial increase followed by a gradual reduction was found in both seawater acclimating and cortisol treated fish. Our data indicated that both CR and NPR-B expression were stimulated by exogenous cortisol treatment and seawater acclimation. The results implied that cortisol should play a role in coordinating the action of natriuretic peptide in gill cells during seawater acclimation. Although the changes in CR and NPR-B were observed in the gill epithelia, information on the differential expression of the receptors in particular cell type was lacking. Hence in the second part of the study, freshwater and seawater gill chloride and pavement cells were isolated using 3-steps percoll gradient centrifugation (Wong and Chan, 1999). Total RNA was extracted for PCR amplification to determine the expression level of the receptors. CR was found in both of pavement (PVC) and chloride (CC) cells while the NPR-B was only detected in chloride cells (fig. 2), indicating the chloride cell functions were mediated by both cortisol and natriuretic peptide (i.e. CNP).



(B)

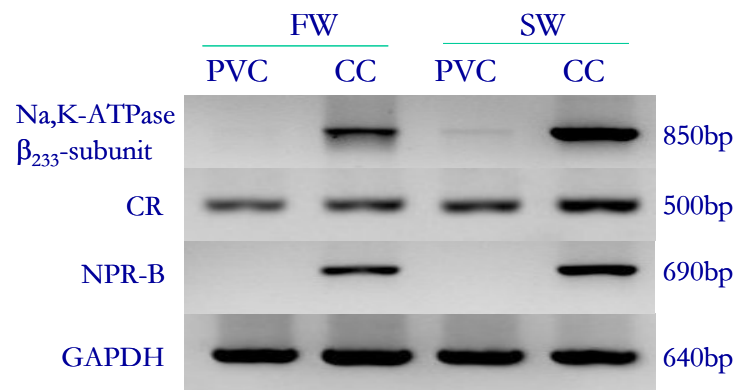
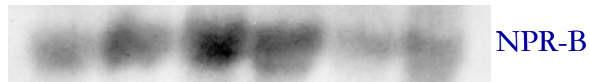
Cortisol treatment on  
freshwater adapted eels

FW day 1 day 2 day 5 SW



Cortisol treatment on  
freshwater adapted eels

FW day 1 day 2 day 5 day 10 SW



**Figure 1.** Comparative analysis on the changes of (A) cortisol receptor (CR) and (B) natriuretic peptide receptor (NPR-B) expressions during seawater acclimation and exogenous cortisol treatment of freshwater fish.

**Figure 2.** PCR results showed the differential expression of Na,K-ATPase  $\beta_{233}$ -subunit isoform, cortisol receptor (CR) and natriuretic peptide receptor (NPR-B) mRNA in isolated freshwater and seawater pavement (PVC) and chloride cell (CC). Na,K-ATPase  $\beta_{233}$ -subunit and NPR-B transcripts were mainly expressed in chloride cells.

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### Acknowledgements

This work was supported by the Research Grants Council, Hong Kong.

**ACTIVATION AND INACTIVATION  
OF MITOCHONDRIA-RICH CELLS  
IN TILAPIA LARVAE  
ACCLIMATED TO AMBIENT CHLORIDE CHANGES**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

Mitochondria-rich (MR) cells in branchial epithelium of freshwater teleost are responsible for absorbing  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  actively from external environment. Resembling to other ion-transporting epithelial cells, MR cells contact with both internal and external milieus by basolateral and apical membrane domains, respectively, and pump the ions transcellularly from environment into blood circulation. Ambient  $\text{Cl}^-$  is suggested to be transported against gradient by  $\text{Cl}^-$  transporters distributed on the apical opening (apical membrane) of MR cells. It is well known that the apical openings of MR cells recessed to form apical crypts in seawater-acclimated fishes, but flush or slightly raised above the adjacent pavement cells in most freshwater-acclimated fishes (reviewed by Perry and Laurent, 1993). However, in the special case of tilapia, *Oreochromis mossambicus*, recessed apical surface of MR cells were also founded in freshwater-acclimated individuals. In our previous study on freshwater tilapia, we categorized these MR cells with different apical surface as wavy-convex, shallow-basin, and deep-hole subtypes (Lee et al., 1996). We found the structure of these apical openings varied with ambient  $\text{Cl}^-$  levels and suggested that the three subtypes of cells representing MR cells equipped with distinct capabilities

of Cl<sup>-</sup> uptake (Lin and Hwang, 2001). Moreover, our evidences implicated that MR cells modulate their apical structure quickly (in hours) to compensate for ambient Cl<sup>-</sup> disturbance. In the present study, we provide further evidences to support our model that MR cells in tilapia can be activated or inactivated to modulate their Cl<sup>-</sup> uptake capabilities through modifying their exposed apical openings.

### Experimental designs and results

Yolk-sac MR cells in tilapia larvae were examined with immunocytochemistry and vital staining during acclimation to high or low ambient Cl<sup>-</sup> levels (High Cl<sup>-</sup>, 10 mM; Low Cl<sup>-</sup>, 0.005 mM; Normal Cl<sup>-</sup>, 0.5 mM). By using Concanavalin-A (Con-A) and Na pump double-staining, the MR cell on yolk-sac were labeled and discriminated to active (Con-A positive) or inactive (Con-A negative) groups. Therefore, the ratios of active MR cells in total labeled MR cells were counted and compared between the 3 Cl<sup>-</sup> acclimating groups. Results showed that ratio of active cells increased gradually during 48 h acclimation to low Cl<sup>-</sup> medium, but declined during acclimation to high Cl<sup>-</sup> medium (fig. 1). However, the total numbers of MR

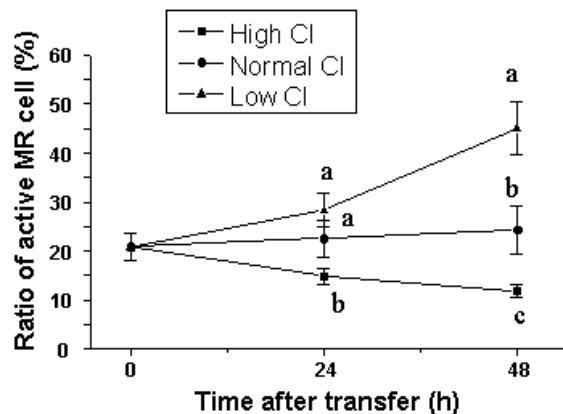


Figure 1. Changes of active MR cell in tilapia larvae acclimated to high, normal, and low Cl<sup>-</sup> (10, 0.5, 0.05 mM) for 48 h. Mean±SD (N=5) is indicated. Different letters indicate significant difference between different groups.

cells were not different between different Cl<sup>-</sup> acclimation groups. Moreover, we also labeled the yolk-sac MR cells with vital staining (DESPEI) and traced the marked MR cells in living larvae. After transfer the labeled larvae from normal water to high or low Cl<sup>-</sup> water, we seriously observed the changes of DESPEI- stained cells every 6 h for 24 h. Under confocal scanning, we found that the cell numbers were maintained

constantly during acclimation, and the newly generated or degenerated cells were less than 10 %. These evidences consistently indicated that no significant turnover of MR cells occurred in larvae during acclimation to high or low ambient Cl<sup>-</sup>. Thus, the dramatic changes of apical openings subtypes within 24 h of acclimation (Lin and Hwang 2001) can be consider as morphological and functional modification of terminal differentiated MR cells. Furthermore, we suggested that MR cells can be activated from a inactive cells that are not contact with external environment and gradually expend their openings to upregulate Cl<sup>-</sup> uptake capability, and vice versa, inactivated through constricting their opening to totally being covered by apical adjacent pavement cells. In addition, we also labeled MR cells with Con-A in living animal to examine the structural changes of apical opening during MR cell activation or inactivation. Interestingly, Con-A labeled vesicles were observed inside MR cells during the acclimation, indicating internalization of apical membrane might be involved in the process of activation or inactivation. In the aspect of physiological significance, our finding reveals that through activation or inactivation of MR cells in short-term regulation, tilapia larvae are capable of maintaining internal Cl<sup>-</sup> constant and resisting to ambient Cl<sup>-</sup> fluctuation.

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**OCCURRENCE AND DEVELOPMENT OF LAMELLAR  
MITOCHONDRIA-RICH CELLS IN GILLS OF FRESHWATER-  
ADAPTED MILKFISH (*CHANOS CHANOS*)**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

Milkfish culture in Taiwan has a history of over 300 years. Every year about 30,000 mt of milkfish were produced. Milkfish are cultured in fresh, brackish, and oceanic waters because they are good regulators and are euryhaline throughout their non-diadromous life cycle (Chen, 1990). Previous studies focused on diadromous (i.e. eel and salmon) and non-diadromous freshwater- and estuarine-resident teleosts, i.e. tilapia and killifish, revealed similar positive correlation between environmental salinity and the biochemical activity of gill  $\text{Na}^+, \text{K}^+$ -ATPase (McCormik, 2001). The milkfish, being a marine inhabitant, is a well-suited subject for experiments of salinity adaptation. To avoid the effects of sexual and seasonal differences in gonadal growth, juvenile milkfish were used in the present study. Since our recent physiological studies found different patterns of sodium pump expression upon salinity challenge in milkfish, it is intriguing to realize the occurrence and development of lamellar mitochondria-rich (MR) cells in gills of freshwater-adapted milkfish (*Chanos chanos*).

**Experimental designs and results**

Distribution of MR cells in freshwater-adapted juvenile milkfish was examined with the light microscope. By using Na pump monoclonal antibody, MR cells were labeled (Dang et al., 2000) and discriminated between lamellae and interlamellar regions of filaments. The number of labeled MR cells on lamellae or interlamellar regions were counted and compared between the seawater and freshwater groups. Our results showed

that the number of lamellar MR cells (LMRCs) increased during long-term freshwater-acclimated milkfish (Fig. 1). The total numbers of MR cells thus were significantly higher in seawater group. We also labeled MR cells of time-course sampled fish with Na pump monoclonal antibody after acclimation to fresh water (0, 6, 12, 24, 48, and 96hrs). LMRCs were first found in 24hrs-group and raised in number in 48hrs-group. Our results revealed that the LMRCs occurred during 24hrs in freshwater-acclimated fish. In order to realize the development LMRCs, the freshwater-acclimated milkfish were injected with Brd-U tracer and sampled at 6, 12, 24, 48, and 96hrs. Double labeling of Na pump and Brd-U will give evidence to the generation of LMRCs. Our preliminary data demonstrated significant increase of LMRCs in freshwater-adapted juvenile milkfish and the milkfish LMRCs occurred in 24hrs after transfer to fresh water. The Brd-U labeled MR cells will also be observed to understand the development of milkfish LMRCs.

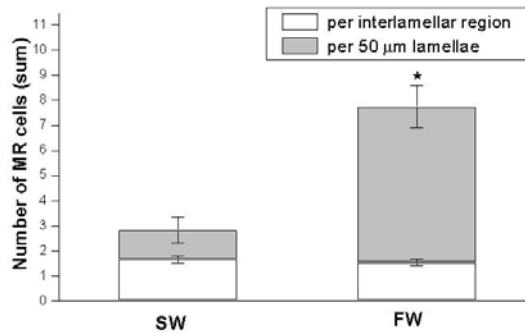


Figure 1. Number of MR cells on gills of seawater and freshwater-adapted milkfish (N=5, Means±SE). Bars in different colors indicated various regions of the gill. \*Significant different, p<0.05, Student's *t*-test.

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**DIVERSITY OF THE MOLECULAR STRUCTURE OF  
STANNIOCALCIN IN THE ACTINOPTERYGIANS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

For some time, stanniocalcin (STC) has been described as a homodimeric glycoprotein that is involved in calcium and phosphate regulation. This hormone was first discovered within small kidney-associated endocrine glands, referred to as the corpuscles of Stannius (CS), that occur only in certain rayfined fishes (Actinopterygii), the holostean and teleostean fishes. Recently, it was revealed that several mammalian tissues also contain STC and that STC mRNA expression is also present in many tissues and organs. Similar to the function in fish, mammalian STC plays a role in mineral metabolism. Primary structures of fish STC have been determined only in basal and generalized teleosts, such as several salmonid species and the Australian eel. Knowledge of the primary structure of STC in both more ancient and more derived actinopterygians would help in our understanding of the evolution of this molecule. At the same time, sequence comparison of STC could assist in our view of the taxonomic relationship of the various groups of fishes. The present report describes recent data that are part of a larger investigation into the molecular evolution of STC among the actinopterygians.

The bowfin (*Amia calva*) is one of two extant holostean species with ancient ancestral links to modern-day teleosts. The bowfin provides us with the opportunity to study STC structure in the most ancient extant species to possess CS. The CS of the bowfin are abundant (several hundred) and scattered throughout much of the kidney, while in the teleostean fishes, the number of CS is low (2-5) and localized (Youson et al., 1976). Osteoglossiformes (bonytongues) is among the most ancient of the teleostean orders. The Osteoglossiformes are believed to have developed in a common

geographical region prior to the separation of the tectonic plates (Li and Wilson, 1996). Currently, the geographical distribution of the species is very diverse. Several osteoglossiforme species of interest to the present study are the elephantnose (*Gnathonemus petersii*) and the butterflyfish (*Pantodon buchholzi*) from Africa, and the silver arawana (*Osteoglossum bicirrhosum*) from South America. The relationship of species within the Osteoglossiformes and the connections of this order to other teleosts are not completely clear (Li and Wilson, 1996). An examination of the STC of these species allows for an evolutionary and comparative analysis of the taxonomic relationships of the individual Osteoglossiformes and with other teleosts. In the present study, we cloned STC cDNAs and compared deduced amino acid sequences from bowfin (holostean) and three species of Osteoglossiformes, elephantnose, butterflyfish and arawana, to obtain further insight into the molecular evolution of the STC gene.

Salmonid and Australian eel STCs and mammalian STC-1 have eleven cysteine residues located in an identical position. High numbers of cysteines provide greater potential for intra- and inter-monomeric disulfide linkages. For example, in chum salmon, five intra-monomeric disulfide linkages exist. One cysteine, which is located closest to the C-terminus of the protein, is unpaired and is involved in the formation of stanniocalcin homodimers (Hulova et al., 1999). The present study revealed that bowfin STC consists of eleven cysteine residues like that of other vertebrate STCs. The bowfin STC shows the highest amino acid sequence identity with the Australian eel (69%), compared to the coho salmon (65%), Osteoglossiformes (59-61%), and mouse STC-1 (56%). Since bowfins are the most ancient of vertebrates known to possess CS and STC, these data suggest that the structure of STC has been well conserved throughout vertebrate evolution. However, the STCs from the three Osteoglossiformes contain only ten cysteine residues because one cysteine is replaced by either arginine or histidine (Fig.1).

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      bowfin: 151 SRLGPMSMLFQLLQSKPCPASSASSTSSA 190
Australian eel: 151 ---E---GV-----T-AC-P-A-GG-GPV 190
      coho salmon: 151 A----D-ET-----N-HC-QG-NQGPN-- 190
      mouse-1: 151 EKI--N-AS--HI--TDHCAQTHPRADFNR 190
silver arawana: 151 A-----VA---K----SSRSG-A-QASNND 190
      elephantnose: 149 -----VA---K----S-RS-PDS-QNKVE 188
      butterflyfish: 151 -----AL--K----S-HT-P-DPALLG- 190

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Fig.1. Sequence comparison of a common C-terminal region in STCs of various fishes and the mouse. Note the cysteine (C) that is critical for the homodimeric molecular STC (box) is substituted (in bold)

type) by either arginine (R) or histidine (H) in the these Osteoglossiforme species. An hyphen (-) represents an identical amino acid to bowfin STC.

The cysteine substitution occurs at the site of inter-monomeric disulfide linkage. Western blot analysis of arawana CS extract with anti-salmon STC revealed a single 21 kDa band under non-reducing conditions, and a single band of 25 kDa under reducing conditions. These data indicate that arawana STC exists as a monomeric peptide but the nature of the STC peptide from the other Osteoglossiformes needs to be confirmed. The STC moleculars from the osteoglossiforme species that have been studied to date seem to be unique among known vertebrate STCs. It remains to be determined whether the monomeric form of STC as describe herein introduces any significant change in known biological activity of CS-generated peptides. Furthermore, in order to obtain further insight into the molecular evolution of STC in actinopterygians, it is important to characterize STC genes from the gar (holostean, order Semionotiformes), and other species of Osteoglossiformes such as mooneye or goldeye from North America.

This study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to JHY

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**MOLECULAR EVOLUTION OF  
NATRIURETIC PEPTIDES  
IN FISH**

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**Introduction**

Natriuretic peptides (NPs) comprise a hormone family that plays a pivotal role in ion and water regulation (Takei, 2000). In tetrapods, three types of NPs are identified; endocrine ANP and BNP secreted from the heart, and paracrine CNP synthesized mostly in the brain. In teleost fish, which are osmoregulators that maintain plasma osmolality at ca. 1/3 of seawater (SW) like tetrapods, three peptides are also identified, endocrine ANP and VNP and paracrine CNP. In elasmobranchs, which are partial osmoconformers that increase plasma osmolality to the SW level by urea and trimethylamine oxide, only CNP is present in the heart and brain (Kawakoshi et al., 2001). It seems therefore that CNP is the ancestral form of the NP family, which has become more complicated in vertebrate in parallel with the evolution of osmoregulatory mechanisms. If this is the case, which type of NPs is present in the hagfish, the most primitive extant vertebrate that is a full osmoconformer? It is also of interest to examine how many types of NPs exist in the sturgeon, a most primitive bony fish that is a full osmoregulator. In the present study, we cloned cDNAs encoding NPs from the heart and brain of hagfish and sturgeon to depict a clearer picture for the evolution of the NP family in fishes.

**Materials and Methods**

The hagfish, *Eptatretus burgeri*, were collected in Koajiro Bay, Kanagawa, and the sturgeon, *Acipenser transmontanus*, were purchased from a commercial source. For tissue sampling, each species was anaesthetized, and the heart and brain were dissected out and frozen quickly in liquid nitrogen. The rapid amplification of cDNA ends (RACE) method was performed for cloning of NP cDNAs. Five degenerate sense primers corresponding to the conserved region of known NP peptides were used for the 3'-RACE. Eel ANP, VNP and CNP were amplified by the primer pairs. For 5'-RACE, specific antisense primers were designed based

on the NP cDNA sequences amplified by the 3'-RACE. In order to amplify minor NPs,				
ab	Human	ANP	SLRRSSCFGGRMDRIGAQSGGLGCNSFRY	ased method
an		BNP	SPKMVQGSQCFGRKMDRISSSSGLGCKVLRHH	vious paper
(K		CNP	GLSKGCFGLKLDRIGSMSGLGC	
	Eel	ANP	SKSSSPCFGGKLDRIGSYSGLGCNSRK-NH <sub>2</sub>	
Rc		VNP	KSFNSCFGTRMDRIGSWSGLGCNSLKNGTKKKIFGN	
		CNP	GNRRCFGLKLDRIGSLGSLGC	
Or				luced amino
ac	Sturgeon	ANP	RGSSGCFGSRIDRIGSMSSMGCGSRK-NH <sub>2</sub>	ig. 1). After
rer		VNP	SMNGCFGNRIERIGSWSSLCNNSRFSGSKKRIF	Thus, hfNP
mk		CNP	QQGRGCFGMKLDRIGSMSGLGC	identified in
ela	novel NP		NTKRYSGCFGRRLDRIGMSALGCNGGSRLSYKRS	akoshi et al.,
20	Dogfish	CNP	GPSRGCFGVKLDRIGAMSGLC	ing the issue
of	Hagfish	NP	LGSTSDGCFGVKMDRIGASTGLGCRGARRRTFS-NH <sub>2</sub>	

Figure 1. Natriuretic peptides identified in mammals (human), teleost fish (eel) and elasmobranchs (dogfish). Sequences of sturgeon and hagfish were obtained in this study. Lines show a S-S bond.

Together with the present data, we can depict a new phylogenetic tree on the molecular evolution of the NP family (Fig. 2). In hagfish and elasmobranchs, only one NP, hfNP or CNP, has been identified. Hagfish has plasma ionic concentration almost identical to SW and thus need no osmoregulation and ionoregulation. Elasmobranchs has plasma osmotic to SW but its plasma ionic concentration is much lower than SW. Therefore, they need ionoregulation but no osmoregulation. Accumulating data indicate that the NP family is essential for adaptation of teleost fish to diverse salinity environments (Takei and Hirose, 2002). It seems therefore that the NP family is not so diversified in animals whose osmoregulatory mechanisms are not well developed. It is known that the sturgeon is a euryhaline species that lives in the estuarine area where dramatic salinity changes occur every day. It seems relevant that four NPs are present in this euryhaline species. It should be determined how many NPs are present in the lamprey, which is another cyclostome species but has highly developed osmoregulatory mechanisms.

Figure 2. Phylogenic distribution of natriuretic peptides in vertebrates.

### Acknowledgements

We thank Misaki Marine Biological Station, the University of Tokyo, for collecting hagfish. We are also grateful to Dr. Y. Takagi of Hokkaido University for helping us sample sturgeon tissues.

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