

## DEVELOPMENTAL CHANGES IN $\text{Na}^+$ , $\text{K}^+$ -ATPASE ACTIVITY IN MOZAMBIQUE TILAPIA (*Oreochromis mossambicus*) EMBRYOS AND LARVAE IN VARIOUS SALINITIES

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### Abstract

Osmoregulation is essential to life in complex organisms. The tilapia, *Oreochromis mossambicus*, is a euryhaline species capable of maintaining hydromineral balance from early stages of development in either fresh water or in seawater. In adult teleost fish, including the tilapia, the gills are a principal site for ion regulation, but in developing embryos, the gills are not yet formed or fully functional. Chloride cells are the main site of ion exchange and are found in rich abundance in the yolk-sac membrane before the gills develop or become functional. This was the first study measuring  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in whole embryos and larvae, isolated larvae, and yolk-sac membranes of the Mozambique tilapia (*Oreochromis mossambicus*) during development in fresh water and seawater. During development, embryos and larvae in seawater had overall higher  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity compared with those in fresh water. Yolk-sac membranes isolated from 0 to 5 day old larvae had higher  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity per mg protein compared with isolated larvae and whole fish. ATPase activity in the yolk-sac membrane decreased during early development (0 to 5 days post hatch) as the yolk-sac was being absorbed and the gills were developing. These results suggest that developing tilapia shift the site of ion exchange from the yolk-sac membrane to the gill, with higher  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in seawater than in fresh water.

### INTRODUCTION

Osmoregulation is the maintenance of ion and water balance in both the intra- and extracellular fluids. Osmoregulation is essential to life and is energetically expensive; fish expend between 20 and 50 % of resting metabolic energy on osmoregulation (Boef and Payan, 2001). When the hydromineral balance is challenged, the organism must respond to reset homeostasis, either by behavioral changes (e.g. increased drinking of seawater, Miyazaki *et al.*, 1998; or moving to a different location, Wolcott and Wolcott, 2001) or by physiological responses (e.g. copious dilute urine output in fresh water; Miyazaki *et al.*, 2001). In humans, pathological consequences such as high blood pressure, renal failure, or death can occur when the osmoregulation fails (Guyton, 1995).

In the gills, chloride cells, also known as mitochondria-rich cells, regulate ion fluxes between the organism and its environment. Chloride cells are the active site in which ions are "pumped" into the cell from the external environment in fresh water (FW) and actively extruded from the cell to the external environment in seawater (SW) (Silva *et al.*, 1977). Energy for these activities is provided by ATP which is converted to ADP providing the energy to transfer sodium from the cell and potassium into the cell.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity increases in the gills when fish are transferred to environments with higher salinities (McCormick, 1993; Shrimpton and McCormick, 1999; Suresh and Lin, 1992).

Mechanisms of osmoregulation in developing embryos and larvae, in which the gills, gut, and kidneys are not yet fully functional, is not well understood. Chloride cells in the yolk-sac membrane have been used as an indicator of osmoregulatory changes between salinities in developing Mozambique tilapia (Ayson *et al.*, 1994; Shiraishi *et al.*, 2001).  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in whole tilapia embryos has, to our knowledge, not been measured.

Mozambique tilapia are a euryhaline species that can survive in both FW and SW (Hwang *et al.*, 1999). Developing tilapia embryos and newly hatched larvae are completely euryhaline and can tolerate direct transfer from FW to SW and vice versa. After yolk-sac absorption fry lose the ability for direct transfer from FW to SW, but retain the ability to acclimate to gradual increases in salinity. Tilapia regain euryhalinity during juvenile stages, but retention of the ability to osmoregulate in SW through adulthood requires regular transfer between FW and SW (Stickney, 1986; Fiess *et al.*, 2007). Interestingly, when tilapia fry, juveniles or adults are maintained for extended periods in SW or in higher salinity, they retain the ability to osmoregulate after direct transfer to FW.

The purpose of this study was to examine the developmental changes in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae reared and/or acclimated to FW and SW. To quantify these changes we measured  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity throughout development comparing whole fish, the isolated larvae, and the yolk-sac membrane. The tilapia is an important aquaculture species. As such, determining the osmoregulatory differences between FW and SW tilapia during early development will provide information useful for improving rearing and production strategies.

## MATERIALS AND METHODS

### *Fish*

Adult tilapia were kept in outdoor 5000 L tanks supplied with a continuous flow of FW under nature photoperiod at the Hawai'i Institute of Marine Biology, Kaneohe, Hawai'i. Water temperature was maintained at  $25 \pm 2$  °C. They were fed once per

day with Silver Cup Trout Feed (Nelson and Sons, Murray, UT) at approximately 2% body weight. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai'i at Mānoa. Eggs and larvae were obtained from FW brooding females during April through August 2007. Females were removed from the tank with a microfine mesh net and the mouth of a female was held open to check for eggs. Eggs or larvae were transferred to 1 L plastic containers with fresh FW. Eggs were either transferred to 100% SW (32 parts per thousand, (ppt)) or retained in FW. Post-hatch larvae were transferred from FW to 50% SW (17 ppt) for 24 h and then acclimated to 100% SW. Eight embryos or larvae were sampled at each time point and individuals were placed in 100  $\mu$ l SEI buffer (250 mM sucrose, 10 mM Na<sub>2</sub>EDTA, 50 mM imidazole) in 500  $\mu$ l Epindorf tubes and stored at -80 °C until analysis.

### ***Dissection***

Very fine forceps and scalpel were used to separate larvae from the yolk-sac on day 0 (day of hatching; Fig. 1), and days 1, 3, and 5 post hatching. A larva was placed in a Petri dish under a dissection microscope and the yolk-sac was separated from the larval body. The yolk-sac membrane was carefully peeled away from the yolk, and the yolk was discarded. Individual larval body and isolated yolk-sac membrane were placed in 100  $\mu$ l SEI buffer in 500  $\mu$ l Epindorf tubes and stored at -80 °C until analysis.

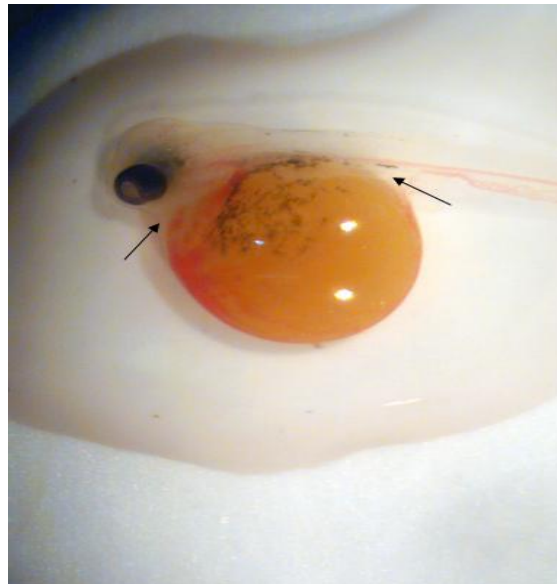


Figure 1. Tilapia larva on day 0 (just hatched). Very fine forceps and scalpel were used to separate larval body from the yolk-sac starting at points indicated by arrows. The yolk-sac membrane was then peeled away from the yolk and the yolk was discarded.

***$\text{Na}^+$ ,  $\text{K}^+$ -ATPase Assay***

$\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was determined following McCormick (1993), modified slightly for use with non-salmonids. As a preliminary validation of the assay, primary gill filaments from adult tilapia in FW and SW ( $n = 3$ ) were analyzed.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was significantly higher in the SW gill compared with the FW gill (FW:  $1.2 \pm 0.6 \mu\text{mol ADP/ mg protein/ h}$ ; SW:  $4.19 \pm 1.5 \mu\text{mol ADP/ mg protein/ h}$ ,  $p < 0.05$ ). These values were in accordance with those obtained in previous studies (Fiess *et al.*, 2007; McCormick, 1993; Hiroi and McCormick, 2007). In a preliminary experiment, various concentrations (20, 10, 5, and 2  $\mu\text{l}$ ) of homogenate (intact fish, isolated larvae, and yolk-sac membrane) were added to the assay to determine the amount of homogenate needed to measure the activity relative to the amount protein per sample. It was determined that 2  $\mu\text{l}$  homogenate for intact fish, and 10  $\mu\text{l}$  homogenate for isolated larvae and yolk-sac membrane would be used throughout the analysis in order to normalize the enzyme activity to the amount of protein per sample.

Samples were thawed immediately prior to assay and all reagents were kept on ice throughout the procedure. SEID buffer (50  $\mu\text{l}$ ; 0.15 g sodium deoxycholic acid in 50 ml SEI buffer) was added to each sample. A hand-held homogenizer (Kontes, Vineland, NJ) was used to homogenize the samples. Samples were centrifuged for 5 min at 12,000  $g$  at 4  $^{\circ}\text{C}$  and were added in quadruplet to a 96-well clear bottom plate (Costar, Corning Inc, NY). Assay mixture (solution A) contained 4 U lactate dehydrogenase (LDH)  $\text{ml}^{-1}$ , 5 U pyruvate kinase (PK)  $\text{ml}^{-1}$ , 2.8 mM phosphoenolpyruvate (PEP), 0.7 mM adenosine triphosphate (ATP), 0.22 mM nicotinamide adenine dinucleotide (reduced form, NADH), and 50 mM imidazole (pH 7.5) (all biochemical reagents from Sigma, St. Louis, MO). Assay solution B was as above but also contained 0.5 mM ouabain. Solution A was added (200  $\mu\text{l}$ ) to half of the wells, and solution B to the other half. Protein content was measured using a Coomassie blue protein assay kit (BioRad, Hercules, CA).  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and protein concentration were measured using a temperature-controlled (25  $^{\circ}\text{C}$ ) plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA) and the computer program SoftmaxPro 4.8 (Molecular Devices).

$\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was calculated as the difference in ATP hydrolysis in the presence and absence of ouabain, expressed as micromoles of ADP per milligram of protein per hour. To compare the enzyme activity per individual in the isolated larvae and yolk-sac membranes, the activity was multiplied by the amount of protein, expressed as micromoles of ADP per individual per hour. Since the amount of homogenate used for the whole fish was 2  $\mu\text{l}$  (i.e. 1/5 of the amount for larvae and

yolk-sac membrane samples) the specific activity was multiplied by 5 and by the amount protein and is expressed as micromoles of ADP per individual per hour. To determine the relative activity in the yolk-sac membrane or isolated larvae on days 0, 1, 3, and 5 post-hatching, the activity in the yolk-sac membrane or larvae was divided by the total activity (the yolk-sac membrane and larvae combined). This value was multiplied by 100 and the final value expressed as percent activity.

### ***Statistics***

This design was a Completely Random Design (CRD). Two-way Analysis of Variance (ANOVA) was performed using JMP IN 5.1 software (SAS, Cary, NC) followed by Least Significant Difference (LSD) test. Dunnett's test was performed to determine significant differences in enzyme activity compared with activity on day -3 (control). P values < 0.05 were considered significant. Calculations were performed using the computer program SoftmaxPro 4.8 (Molecular Devices) and EXCEL (Microsoft Office). Results are presented as means  $\pm$  standard errors of the mean.

## **RESULTS**

### ***Changes in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity during the course of development***

Eggs and larvae that were fertilized in FW and transferred to SW before hatching exhibited 100% survival, but post-hatch larvae and fry did not tolerate direct transfer to SW. When post-hatch larvae were transferred first from FW to 50% SW for 24 h and then acclimated to 100% SW, no mortality occurred.

Figure 2 shows changes in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity of the whole body, expressed per individual, during development in FW or in SW. There was no significant difference in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity between FW and SW-acclimated fish on day -3 (2 days before hatching). On subsequent days, the enzyme activity was significantly higher in SW-acclimated fish compared with FW fish. There was no change in the activity in the embryos, larvae and fry in FW, whereas in those kept in SW, a peak in activity was observed 1 day after hatch, and declined gradually and significantly until day 12 after hatch. When enzyme activity was compared with the control (day -3) there was a significant elevation on days -2 and 5 after hatching in FW and significant elevations in activity on days -2, -1, 1, 2, and 5 in SW (Dunnett's test, Fig. 2). Essentially similar results were obtained when the enzyme activity was expressed as per mg protein (data not shown).

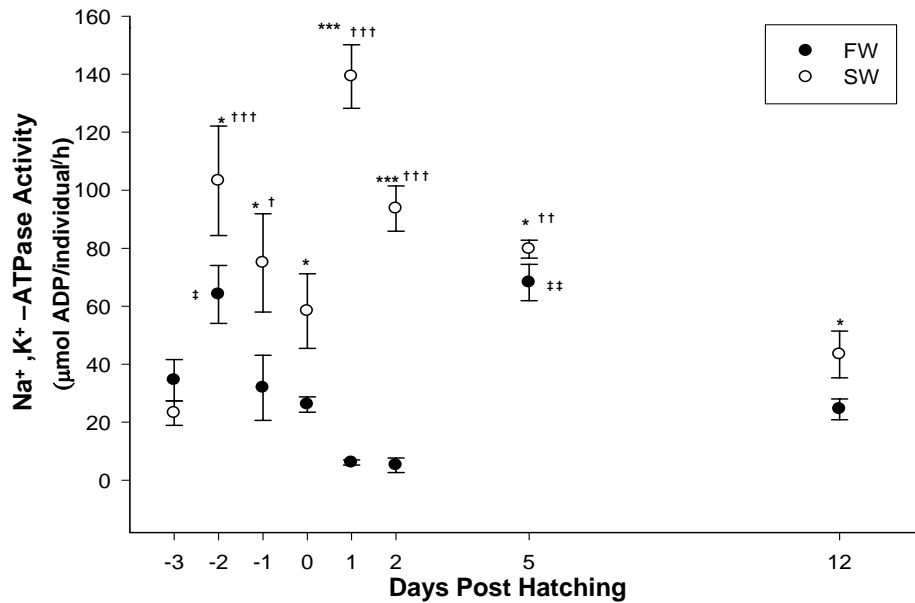


Figure 2.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity of the whole body in freshwater (FW)- and seawater (SW)-acclimated fish during the course of early development. Enzyme activity was expressed as  $\mu\text{mol ADP/individual/h}$ . Values are mean  $\pm$  S.E.M,  $n = 7-8$ . \*, \*\*\* Significantly different from FW at  $P < 0.05$  and  $0.001$ , respectively. †, ††, ††† Significantly different from day -3 in SW at  $P < 0.05$ ,  $0.01$ , and  $0.001$ , respectively. ‡, †† Significantly different from day -3 in FW at  $P < 0.05$ , and  $0.01$ , respectively.

### *Changes in tissue distribution of $\text{Na}^+$ , $\text{K}^+$ -ATPase activity during development*

Figure 3 shows distribution of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity, expressed per individual in larvae 0, 1, 3 and 5 days post hatching in FW and SW.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was significantly higher in fish developing in SW than those in FW except on day 5 after hatching. Enzyme activity in the whole fish significantly increased during development in both FW and SW from day 0 to day 5. The percent  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity contained in the yolk-sac membrane of FW-acclimated fish significantly decreased from 83% of total body activity on day 0 to 49% on day 5. Enzyme activity in the yolk-sac membrane decreased in SW-acclimated fish from 69% on day 0 to 61% on day 5, however this was not statistically significant (Fig. 3). Concurrently, percent  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in the larval body increased proportionately from day 0 to day 5 indicating a shift in the site of ion exchange away from the diminishing yolk-sac membrane to the body. It is possible that the enzyme activity is being concentrated in the developing gills; however  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in the early-stage larval gills was not determined in this study (Li *et al.*, 1995).

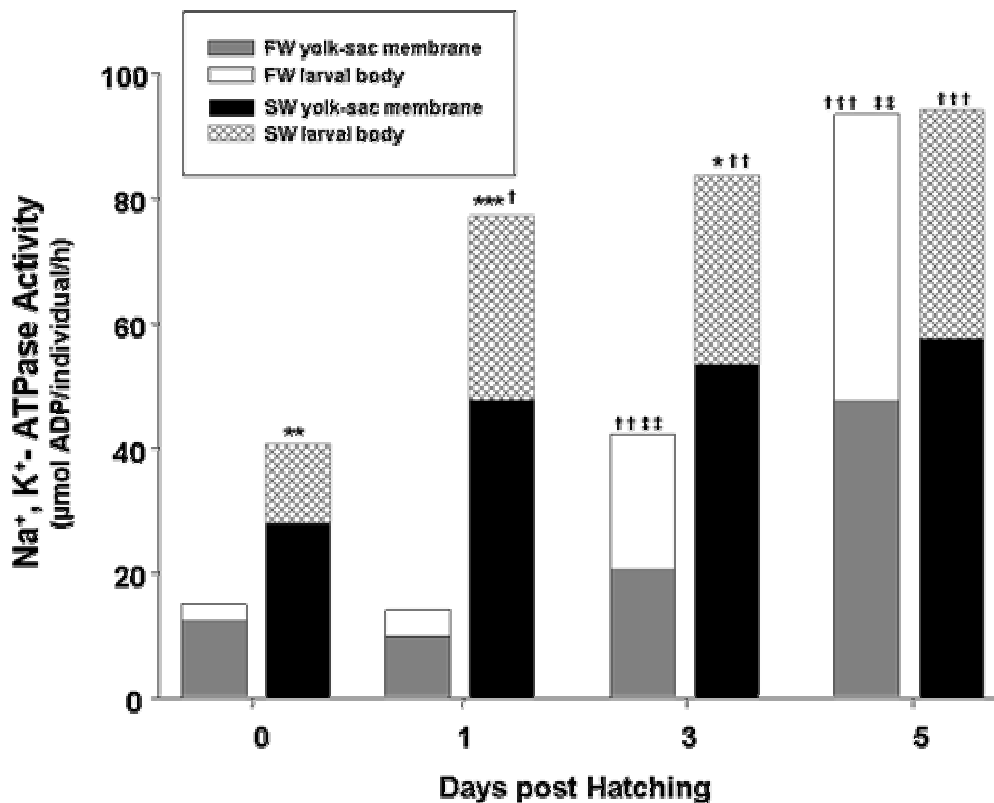


Figure 3. Na<sup>+</sup>, K<sup>+</sup>-ATPase activity of tilapia larvae 0, 1, 3, and 5 days post hatching, expressed per individual in fresh water (FW) and seawater (SW). Enzyme activity in isolated larvae and yolk-sac membrane are expressed as percent of whole body (larvae or yolk-sac membrane / (yolk-sac membrane + larvae)). \*, \*\*, \*\*\* Significantly different from FW at P < 0.05, 0.01, and 0.001, respectively. †, ††, ††† Significantly higher whole body activity than on day 0 at P < 0.05, 0.01, and 0.001, respectively. †† Significantly lower proportional activity in yolk-sac membrane compared with day 0 at P < 0.01. n = 7-8.

Figure 4 indicates the distribution of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in the whole body, isolated larval body, and yolk-sac membrane as expressed as  $\mu\text{M ADP per mg protein per h}$ . In larvae on the day of hatching (day 0), the enzyme activity was significantly higher in yolk-sac membrane and larval body compared with the whole larvae including yolk. Enzyme activity was also significantly higher in SW-acclimated larvae compared with FW-acclimated larvae on day 0. On day 1 after hatching, enzyme activity was significantly higher in yolk-sac membrane compared with larval body or whole larvae and activity was significantly higher in SW compared with FW. On days 3 and 5, a similar pattern was observed with enzyme activity in SW yolk-sac membrane significantly higher than in FW or in larval body or whole larvae. Enzyme activity per mg protein was highest in the yolk-sac membrane, lowest in the whole fish, and decreased from day 0 to day 5 (Figure 4).

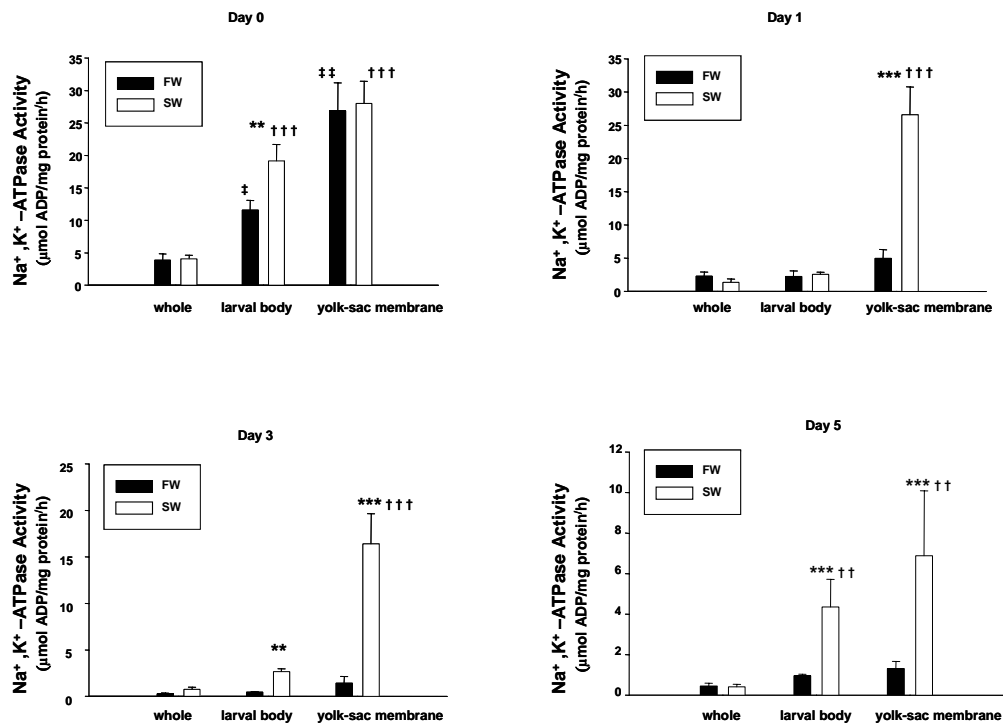


Figure 4.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity of tilapia larvae 0, 1, 3, and 5 days post hatching, expressed as per mg protein, in whole body, isolated larvae, and yolk-sac membrane in fresh water (FW) and seawater (SW). Values are mean  $\pm$  S.E.M,  $n = 7-8$ . \*, \*\*, \*\*\* Significantly different from FW at  $P < 0.05$ ,  $0.01$ , and  $0.001$ , respectively. ††, ††† Significantly different from whole body in SW at  $P < 0.01$ , and  $0.001$ , respectively. † Significantly different from whole body in FW at  $P < 0.05$ .

## DISCUSSION

Osmoregulation is necessary for the proper function and survival of both FW- and SW-acclimated fish. The mechanisms that control osmoregulation in embryonic and larval fish have recently been the focus of much study as the gills, gut, and kidney are not fully formed or functional (for review: Varsamos *et al.*, 2005). Since the embryos and larvae are able to maintain osmoregulatory homeostasis before functional gills develop (Varsamos *et al.*, 2005), the integument and yolk-sac membrane are likely the main sites of osmoregulation during the embryonic and larval stages. This report is the first to investigate changes in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in embryonic and larval tilapia during the course of early development.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was significantly higher in SW fish compared with FW fish. These results are consistent with previous observations in adult fish of this species (Fiess *et al.*, 2007; Uchida *et al.*, 1996).

In the present study,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was highest in the yolk-sac membrane, where chloride cells are most numerous before the gills are completely formed or functional (Hiroi *et al.*, 2005; Ayson *et al.*, 1994). Further, enzyme activity

in the yolk-sac membrane declined as development progressed (Fig. 3 and Fig. 4). The combination of larger cell size (Ayson *et al.*, 1994) and higher Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (current study) suggests that the yolk-sac membrane is the principle site of ion extrusion in SW-acclimated tilapia during the early stages of development prior to gill formation and function. These data also suggest that during early development, the decline in activity in the yolk-sac membrane together with the rise in activity in the larval body indicates a developmental shift in the locus of osmoregulatory capacity that results from yolk-sac absorption and the development of new chloride cells in the gills.

The hormonal control of osmoregulation could also be influencing the changes in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity. Studies have shown that cortisol increases Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in Mozambique tilapia (*Oreochromis (Sarotherodon) mossambicus*, Dange, 1986), juvenile rainbow trout (*Onchorhynchus mykiss* (Walbaum), Shrimpton and McCormick, 1999), and salmonids including Coho salmon (*O. kisutch*), Atlantic salmon (*Salmo salar*), Chum salmon (*O. keta*), and Chinook salmon (*O. tshawytscha*) (McCormick *et al.*, 1991). Shiraishi *et al.* (1999) studied the changes in prolactin- and cortisol-receptor gene expression in Mozambique tilapia embryos in FW and SW. Chloride cell differentiation in embryonic tilapia was also studied with changes in salinity and cortisol (Shiraishi *et al.*, 2001). It would be of interest to study the gene expression profiles of the osmoregulatory hormones prolactin and growth hormone in concert with Na<sup>+</sup>, K<sup>+</sup>-ATPase activity during the course of tilapia development to determine some of the other osmoregulatory mechanisms at play during this period.

In conclusion, these results show that tilapia embryos and larvae developing in SW have higher Na<sup>+</sup>, K<sup>+</sup>-ATPase activity compared with their FW counterparts. Na<sup>+</sup>, K<sup>+</sup>-ATPase activity changes over the course of development; it originates in the yolk-sac membrane, where there are high populations of metabolically active mitochondria-rich cells, and subsequently decreased in the yolk-sac membrane during development. Concurrently, enzyme activity increased in the larval body suggesting development of novel chloride cells in the gills as they become functional. We cannot conclude that the increased enzyme activity was located specifically or only in the developing gills as these tissues were not analyzed separately. It is possible that the increase in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in the larval body was also located in the integument, developing intestines, or kidney; however dissection and isolation of these tissues, along with localization techniques would be required. The results of this study improve our understanding of the underlying osmoregulatory mechanisms used by developing tilapia and provide data for further studies aimed at understanding the optimal conditions for maximal rearing and grow-out of this important aquaculture species.

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