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Effects of Cortisol on Ion Regulation in Developing Tilapia
(Oreochromis mossambicus) Larvae on Seawater Adaptation

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ABSTRACT

The yolk diameter of cortisol-treated tilapia (Oreochromis mossambicus) larvae, immersed in freshwater (FW) containing 10 mg L⁻¹ cortisol from 48 h postfertilization to 12 d posthatching, was significantly larger than that of control larvae after 8 d of treatment, suggesting that inhibition on larval growth occurred only after a long-term treatment with cortisol. Tilapia embryos or larvae treated with 1–10 mg L⁻¹ cortisol for 1–2 d and then transferred to 20–30 g L⁻¹ seawater (SW) showed reduced cumulative larval mortality in SW compared with controls. Moreover, 4–5 d of cortisol treatments signiﬁcantly diminished the degree of increase in larval body Na content after the transfer to SW. Significant effect of cortisol on body Na content of larvae occurred as early as 4–8 h after the transfer to SW, while no significant difference was found in the ouabain binding of yolk-sac epithelia between control and cortisol-treated larvae even 12 h after the transfer. Cortisol may be involved in the early phase of SW adaptation in developing larvae, and this mechanism may be achieved by other means than increasing the Na-K-ATPase of yolk-sac epithelia.

Introduction

Body fluids of embryos and larvae of several teleosts, whose gills or kidneys are poorly or underdeveloped, have been found to be able to maintain constancy in ion concentrations and osmolality of their body fluids (Alderdice 1988). The outer membrane of teleostean eggs, the chorion, is permeable to both salts and water but not to larger molecules. Thus, the vitelline membrane is the only effective barrier between embryos and the external medium (Eddy 1982). Embryos and larvae, as well as adults, must adapt to the environments of either hypertonic seawater (SW) or hypotonic freshwater (FW). Recent studies have demonstrated the ion-regulation abilities in embryos and larvae, that is, active secretion of NaCl in seawater (Guggino 1980a) and absorption of Na⁺ (McWilliams and Shephard 1989) and Ca²⁺ (Hwang et al. 1994, 1996) in FW via mitochondrion-rich skin cells (Hwang and Hirano 1985; Hwang 1990; Hwang et al. 1999). Moreover, the mechanism of water balance was also found to be developed at early developmental stages in teleosts (Guggino 1980b; Tytler and Blaxter 1988). How these mechanisms are controlled in early developmental stages of teleosts has not been established.

Cortisol is one of the major corticosteroids in teleosts and has both glucocorticoid and mineralocorticoid functions. Cortisol is thought to be a seawater-adapting hormone because it promotes salt excretion in hypo-osmoregulating fish (Scheer and Langford 1976; Richman and Zaugg 1987; Madsen 1990). This function has been shown to work through the stimulation of gill Na⁺-K⁺-ATPase activity and the development of branchial mitochondria-rich cells (McCormick 1990, 1995; Madsen 1990). Recent studies have demonstrated the presence of cortisol in embryos and larvae whose interrenal tissues are not yet developed (De Jesus et al. 1991; Evelyn et al. 1992; Hwang et al. 1992). Hwang and Wu (1993) proposed that cortisol is involved in hypo-osmoregulation in early developmental stages as well as in the tilapia adult. So far only a few studies have tested this hypothesis. Hwang and Wu (1993) reported that administration of cortisol could increase the survival of tilapia larvae after transfer from FW to SW. Ayson et al. (1995) showed that immersion in cortisol stimulated the size and number of yolk-sac mitochondria-rich cells in FW tilapia larvae.

The purpose of this article was to study the role of cortisol in the hypo-osmoregulatory mechanisms in early developmental stages of fish. Tilapia (Oreochromis mossambicus), a euryhaline species, were selected because their embryos and larvae are able to survive in either FW or SW (Hwang et al. 1999). Changes in mortality, body weight, water content, sodium content, and ouabain binding of yolk-sac epithelia in control and cortisol-treated tilapia larvae after transfer from FW to SW were examined to test if the exogenous cortisol can enhance the hypo-osmoregulatory capacity of tilapia larvae.
Figure 1. Cortisol content in FW tilapia larvae immersed in different concentrations of cortisol from hatching to 3 d posthatching. Mean ± SD (n = 4–5) are indicated. One-way ANOVA (Tukey’s pairwise comparison) was run. Different letters indicate significant differences.

Material and Methods

Animals

Mature tilapia Oreochromis mossambicus from the Tainan Branch of the Fisheries Research Institute were reared in circulating FW at 26°–28°C using a photoperiod of 12 h of light. Fertilized eggs were retrieved from the mouth of a female approximately 48 h before hatching. Embryos were incubated in well-aerated FW at the same conditions as those of adults. The incubation water was changed daily to control the water quality. Tilapia embryos generally took approximately 4 d until hatching under the above conditions. During all experiments, no tilapia larvae were fed. Sampled larvae were anesthetized with tricaine methanesulfonate (MS222) before being subjected to the following measurements.

Cortisol Content

Extraction and measurement of whole-body cortisol in tilapia larvae followed protocols described previously (Hwang et al. 1992). Briefly, larvae (20 individuals were pooled as a sample) were homogenized in phosphate-buffered saline-gelatin (PBSG; pH 7.0) and then extracted with diethyl ether (Merck, Darmstadt). The final extracts were reconstituted with PBSG for radioimmunoassay (RIA) of cortisol. The cortisol antiserum, which was established in our laboratory, showed 10.0%, 1.0%, and 0.01% cross reactivity with 17-α-hydroxyprogesterone, progesterone, and corticosterone, respectively. Cortisol was measured using RIA as described by Hwang et al. (1992). Labeled cortisol (100 μL; 0.1 μCi mL⁻¹) was added to the homogenate in a separate series of tubes before extraction to evaluate extraction efficiency. Recovery rates of extracted cortisol were 82%–89%. Intra-assay variation was 3.6% (n = 8) and interassay variation was 9.4% (n = 8).

Body Weight, Yolk Diameter, and Water Content

After anesthetization, larvae were rinsed with double-deionized water, wiped off with filter paper, and then subjected to the measurements of other features. Yolk diameter of larvae was measured under a dissecting microscope equipped with an ocular micrometer. Larvae were weighed before and after complete dehydration to obtain wet weight (WW) and dry weight (DW). Complete dehydration was obtained in a 100°C oven for approximately 24 h until the dry weight of a larva was constant. Water contents were obtained by the following formula: [(WW – DW)/WW] × 100%.

Body Sodium Content

Larvae were digested with 13.1 N HNO₃ at room temperature overnight. Digested solutions were diluted with double-deionized water and were subjected to atomic absorption spectrophotometry (Z-8000, Hitachi, Tokyo) to measure Na⁺ concentrations. Na⁺ standard solutions from Merck were used as standard curves for the measurements. Matrix effects were calibrated by standard addition.

Ouabain Binding

Yolk-sac epithelia of tilapia larvae were collected by dissection on ice under a stereomicroscope and were immediately homogenized with a motorized Teflon pestle at 600 rpm for 20 strokes in homogenization solution (25 mM Tris-HCl, 0.25 mM

Figure 2. Effect of exogenous cortisol on WW of FW tilapia larvae. Larvae were immersed in cortisol medium from 48 h postfertilization to 12 d posthatching. Mean ± SD (n = 10) are indicated. Student’s t-test was run between control and cortisol groups at each sampling time. Asterisks indicate significant differences (P < 0.05).
sucrose, 2 mM Na$_2$EDTA, protease inhibitors, pH 7.5). The homogenate was centrifuged 6,000 g at 4°C for 20 min, and the supernatant was subjected to ouabain-binding assay. The assay of ouabain binding followed previous methods (Hossler et al. 1979) with some modifications. Supernatant of yolk-sac epithelia was added separately into the reaction medium (3 mM ATP, 6 mM MgCl$_2$, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.5) as a total binding reaction and into nonspecific binding medium (the reaction medium plus 5 mM Na$_2$EDTA and 6.25 mM KCl) as a nonspecific binding reaction. The 3H-labeled ouabain (10 nM reaction $^2$; 49 Ci mmol$^{-1}$) was added into the above reactions just before incubation. The total concentration of ouabain (labeled plus unlabeled) in the total binding reaction was 50 µM. In the nonspecific binding reaction, 1 mM ouabain was added into the nonspecific binding medium as described above. The assays were carried out in triplicate and incubated at 37°C for 1 h. The reaction was stopped by chilling on ice, and the reacted product was collected on a polyethylenimine-pretreated glass microfiber filter (GF/B, Whatman, Maidstone, U.K.) by vacuum filtration (Sampling Manifold 1225, Millipore, Bedford, Mass.) to separate bound and free 3H-ouabain. The filter was mixed with Cocktail T (BDH, Poole, U.K.) and was counted on a β-counter (LS6500, Beckman, Fullerton, Calif.). Protein concentrations of the samples were determined with a protein assay kit (Bio-Rad, Hercules, Calif.) using bovine serum albumin as a standard. Specific binding was obtained by subtracting nonspecific binding from total binding. Each determination was the average of triplicate assays of a pooled sample (with 20 individuals for every sample).

**Cortisol Immersion Experiments**

Cortisol (hydrocortisone, 21-hemisuccinate; Sigma) was dissolved in local tap water to give 1, 5, and 10 mg L$^{-1}$ immersing solutions. Tilapia embryos were incubated in the cortisol immersion solutions as described below, and the immersion solutions were changed daily in order to maintain the cortisol concentration and water quality.

**Experiment 1**

At 48 h postfertilization, FW embryos were immersed in 0 (control), 5, and 10 mg L$^{-1}$ cortisol solutions until 1 d after hatching (total incubation time = 3 d), and then were sampled to measure cortisol content. Ten larvae were pooled into every sample, and a total of 40–50 individuals for each test concentration were sampled.

**Experiment 2**

At 48 h postfertilization, FW embryos were immersed in 0 (control) and 10 mg L$^{-1}$ cortisol solutions until 12 d after hatching (total incubation time = 14 d). Samples were collected daily from 0–12 d after hatching, and 10 individuals for each test concentration and for each sampling time were measured for total length, yolk diameter, body weight, and water content.

**Experiment 3**

At 48 h postfertilization, FW embryos were immersed in 0 (control), 1, and 10 mg L$^{-1}$ cortisol solutions until hatching (total incubation time = 2 d), and then the newly hatched larvae were transferred to 20 g L$^{-1}$ SW for 12 h and subsequently to 30 g L$^{-1}$ SW for another 24 h. Changes in cumulative mortality after transfer to SW were recorded.
Figure 5. Effect of exogenous cortisol on yolk diameter of FW tilapia larvae. Larvae were immersed in cortisol from 48 h postfertilization to 12 d posthatching. Mean ± SD (n = 10) are indicated. Student’s t-test was run between control and cortisol groups at each sampling time. Asterisks indicate significant differences (*P < 0.05).

**Experiment 4**

Newly hatched FW tilapia larvae were immersed in 0 (control), 1, and 10 mg L⁻¹ cortisol solutions for 1 d, and then the larvae were transferred to either 26 or 30 g L⁻¹ SW. Changes in cumulative mortality after transfer to SW were recorded.

**Experiment 5**

At 48 h postfertilization, FW embryos were immersed in 0 (control) and 10 mg L⁻¹ cortisol solutions until 3 d after hatching (total incubation time = 5 d), and then the 3-d-posthatching larvae were transferred to either 24, 27, or 30 g L⁻¹ SW. Changes in body Na⁺ content of larvae after SW transfer were determined. Samples consisted of 5–10 larvae for each test condition and for each sampling time were sampled.

**Experiment 6**

At 48 h postfertilization, FW embryos were immersed in 0 (control) and 10 mg L⁻¹ cortisol solutions until 2 d after hatching (total incubation time = 4 d), and then the 2-d-posthatching larvae were transferred to 24 g L⁻¹ SW. Changes in body Na⁺ content and ouabain binding of yolk-sac epithelia of larvae after SW transfer were examined. Five to 10 larvae for each test condition and for each sampling time were sampled for Na⁺ content, while 60 larvae (20 larvae for a pooled sample, n = 3) were tested for ouabain binding.

**Experiment 7**

Two-day-posthatching FW tilapia larvae were transferred to either FW (control) or 24 g L⁻¹ SW. Similar to experiment 6, changes in body Na⁺ content and ouabain binding of yolk-sac epithelia of larvae after SW transfer were examined.

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**Results**

**Cortisol Immersion Experiments**

Immersion in 5 and 10 mg L⁻¹ cortisol for 3 d resulted in a significant increase in tissue cortisol contents of tilapia larvae (experiment 1, Fig. 1). This effect was dose related; tissue cortisol of 5 and 10 mg L⁻¹ cortisol groups was approximately 6.6- and 8.5-fold higher than that of the control group, respectively, indicating that exogenous cortisol does accumulate in the bodies of larvae.

Body WW and DW, as well as water content, were not significantly affected by long-term (14 d) treatment with cortisol (experiment 2, Figs. 2, 3, and 4). Throughout the 14 d of treatment, only the WW on day 6 posthatching and water content on day 4 posthatching were significantly different (Student’s t-test, *P < 0.05) between the control and cortisol-treated groups. However, the yolk diameter of cortisol-treated larvae was significantly larger (Student’s t-test, *P < 0.05) than that of control larvae from day 6 after hatching, that is, 8 d after treatment (experiment 2, Fig. 5).

**Cortisol Immersion and SW Transfer Experiments**

Based on these results, remaining experiments were conducted with shorter-term (1–5 d) cortisol treatments to avoid significant impact on larval development and growth. After transfer from FW to 20 g L⁻¹ SW for 12 h, tilapia larvae showed approximately 30% cumulative mortality, while almost all of those treated with either 1 or 10 mg L⁻¹ cortisol for 2 d survived (experiment 3, Fig. 6). After subsequent transfer to 30 g L⁻¹ SW, all fish in both control and 1-mg L⁻¹ cortisol groups died at the end of the experiment, but those in the 10-mg L⁻¹ cortisol group had <20% cumulative mortality (Fig. 6).

In the subsequent experiment, 1-d treatment also showed significant and dose-related improvements in the survival of
The body Na\(^+\) content in tilapia larvae increased during the initial 8 h in SW and then declined to near the level of the FW control in the absence of cortisol treatment (experiment 7, Fig. 10). However, it was not until 24 h after the transfer that the yolk-sac epithelial ouabain binding in nontreated SW larvae was significantly higher than that in the FW control.

**Discussion**

Our results show that treatment of tilapia larvae with cortisol at a dose below that causing inhibitory effects on larval development enhances their hypo-osmoregulation ability by improving larval survival and diminishing the degree of increase in body Na\(^+\) content upon SW challenge. This mechanism may be achieved by other means than increasing the Na-K-ATPase of yolk-sac epithelia.

Recent studies have demonstrated the presence of cortisol in fish embryos and larvae whose interrenal tissue is not yet developed (De Jesus et al. 1991; Evelyn et al. 1992; Hwang et al. 1992). The content of maternal origin cortisol in fish eggs appears to decline rapidly, dropping approximately 90% between fertilization and hatching, after which the cortisol in fish larvae increases rapidly during development (De Jesus et al. 1991; Evelyn et al. 1992; Hwang et al. 1992). These results imply that there must be some physiological significance for the presence of cortisol in embryonic and larval stages. Some recent studies indicate that the increased cortisol in developing larvae may be associated with the development of the hypothalamic-pituitary-interrenal axis and the capacity to respond to stress (Barry et al. 1995; Stouthart et al. 1998). In mammals, most of the cortisol in the fetal plasma is maternal in origin until the end of pregnancy (Dalle and Delost 1979; Hennessy et al. 1982). Glickman and Challis (1980) suggested that the cortisol...
Figure 9. Effect of exogenous cortisol on body Na\(^+\) content and yolk-sac epithelial ouabain binding of tilapia larvae after transfer from FW to SW. Embryos at 48 h postfertilization were immersed in 0 or 10 mg L\(^{-1}\) cortisol medium for 4 d and then (2 d posthatching) transferred to 24 g L\(^{-1}\) SW. Mean ± SE (n = 5–10 for Na\(^+\) content, n = 3 for ouabain binding) are indicated. Student's t-test was made between control and cortisol groups at each sampling time. Asterisks indicate significant differences (\(P < 0.05\)).

secreted by the fetal adrenal gland triggers the onset of parturition and provides a stimulus for the maturation of different fetal organ systems. Cortisol appears also to be involved in the development and growth of fish larvae. Cortisol was suggested to enhance the stimulatory action of thyroid hormones on larval development and growth (De Jesus et al. 1991). However, exposure to high levels of cortisol induces deleterious effects in fish (Carragher et al. 1989). Mathiyalagan et al. (1996) showed that cortisol at 5 mg L\(^{-1}\) retarded the development and growth of newly hatched tilapia larvae after 2 wk of exposure. In this study, an inhibitory effect of 10 mg L\(^{-1}\) exogenous cortisol on larval development and growth appeared when the treatment was longer than 8 d.

Exogenous cortisol may affect the development of osmoregulatory mechanisms and, consequently, the physiological features associated with ion and osmoregulation. Freshwater-adapted salmon and rainbow trout juveniles implanted with 80 mg kg\(^{-1}\) cortisol for 3 wk developed a greater drinking rate than did sham fish following transfer to SW (Fuentes et al. 1996). Hwang and Wu (1993) reported that administration of cortisol at 150 mg kg\(^{-1}\) for 8–12 d could increase the survival of tilapia larvae after transfer from FW to SW. Ayson et al. (1995) found that immersion of FW tilapia larvae in 0.1–100 mg L\(^{-1}\) cortisol for 3–9 d stimulated the size and number of yolk-sac mitochondria-rich cells. These previous studies provide no data about changes in body size or other developmental parameters of larvae or juveniles during the experiments. Thus, it is difficult to determine the extent to which the data of these earlier studies can be related to the developmental effects induced by the long-term treatment with exogenous cortisol. On the other hand, this study, by using a cortisol dose that avoided causing inhibitory effects on larval development, demonstrates

Figure 10. Changes in body Na\(^+\) content (a) and yolk-sac epithelial ouabain binding (b) of tilapia larvae after transfer from FW to SW. Larvae at 2 d posthatching were transferred from FW to FW (control) or 24 g L\(^{-1}\) SW. Mean ± SE (n = 6–10 for Na\(^+\) content, n = 4 for ouabain binding) are indicated. Student's t-test was run between the two groups at each sampling time. Asterisks indicate significant differences (\(P < 0.05\)).
a positive role for cortisol in the hypo-osmoregulation of developing larvae.

Cortisol is generally considered a SW-adapting hormone in teleostean fishes. For most studies, treatments of exogenous cortisol with several doses for several days to weeks induced stimulation of differentiation of gill mitochondria-rich cells, activity of gill Na-K-ATPase, and hypo-osmoregulation (see review in McCormick 1995). Dose and timing of the treatments appear to be important for this stimulation. Madsen (1990) reported that repeated injections of 10 µg g⁻¹ cortisol for 2 wk stimulated gill Na-K-ATPase activity and reduced plasma Na⁺ and Cl⁻ levels in rainbow trout (Salmo gairdneri) after transfer to SW, while 0.05–0.1 µg g⁻¹ showed no effect. However, as McCormick (1995) indicated, a possible rapid action of cortisol on ion transport should not be discounted, and only a very few studies have so far discussed this rapid action of cortisol in teleosts. Forrest et al. (1973) found that American eel (Anguilla rostrata), treated with cortisol for 2 d, increased Na⁺ efflux after SW transfer. This effect occurred before an increase in gill Na-K-ATPase activity, which occurred after 14 d of cortisol treatment. Forrest et al. (1973) suggested an additional factor may be involved in the control of Na⁺ efflux. Indeed, an early biochemical response to aldosterone in mammalian renal cells is to increase Na⁺ retention. In these responses, an enhanced Na⁺ influx through the apical Na⁺ channels seems to be followed by an activation of preformed Na-K-ATPase, which enhances Na⁺ transport across basolateral cell membranes (Minuth et al. 1987). According to Ewart and Klip (1995), the long-term actions of aldosterone and other hormones on Na-K-ATPase is mediated by changes in gene expression (and thus, synthesis of new pump units). However, the rapid action of aldosterone in some tissues involves change in the subcellular distribution of Na-K-ATPase units (Ewart and Klip 1995). In this study, treatment as brief as 1–5 d induced an improved hypo-osmoregulation ability in tilapia larvae, that is, it reduced mortality and body Na⁺ content upon SW transfer but did not change the ouabain binding in the yolk-sac epithelia before the body Na content began to decrease. Further studies examining changes in the activity of yolk-sac Na-K-ATPase should be done in order to confirm if short-term actions of cortisol are mediated by activating the already existing Na-K-ATPase in the yolk-sac epithelia of tilapia larvae.

Another possible explanation should be considered. As tilapia larvae rapidly increase body Na⁺ content during development (Hwang et al. 1994, 1995), one of the short-term functions of cortisol may be in regulating Na⁺ influx upon SW transfer. This mechanism does not appear to be mediated by stimulating Na-K-ATPase activity or by increasing the number of pump units. Results of the SW transfer experiment (experiment 7, without cortisol treatment) support the above hypothesis. Upon SW transfer, initially increased body Na content began to decline after 8 h, while it was not until 24 h that a significant increase in yolk-sac epithelial ouabain binding occurred. This suggests that some pathways other than increasing the sodium pump units in yolk-sac epithelia are involved in the early phase of SW adaptation in developing tilapia larvae. However, this conclusion needs further study for confirmation.

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