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Changes in Expression of Prolactin- and Cortisol-receptor Genes during Early-life Stages of Euryhaline Tilapia (*Oreochromis mossambicus*) in Fresh Water and Seawater

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ABSTRACT—Expression of prolactin receptor (PRLR) and cortisol receptor (CR) mRNAs was examined during early-life stages of euryhaline Mozambique tilapia (*Oreochromis mossambicus*) by competitive reverse transcription-polymerase chain reaction (cRT-PCR). Concentration of prolactin receptor mRNA was higher in the gills of mature fish reared in fresh water (FW) than in those reared in seawater (SW), whereas no difference was seen in CR mRNA. Whole eggs just after fertilization contained the receptor mRNAs for both prolactin and cortisol. The concentration of PRLR mRNA increased gradually as the embryo grows both in FW and in SW. On the other hand, the concentration of CR mRNA was highest in the egg just after fertilization, decreased rapidly toward hatching, and increased slightly thereafter. When embryos 3 days before hatching were transferred to SW, the levels of PRLR mRNA were significantly lower at the time of hatching and also 3 days after hatching than in the embryo and larvae maintained in FW. Environmental salinity did not affect CR mRNA content at any stage examined. Both PRLR and CR mRNAs were identified in the yolk-sac membrane and in the embryonic body. Significantly more PRLR gene was expressed in the embryonic body developing in FW than in SW, whereas no difference was seen in the yolk-sac membrane. The greater expression of PRLR gene in embryos and larvae developing in FW than in those in SW clearly indicates the presence of regulatory mechanisms of gene expression in early-life stages of tilapia.

INTRODUCTION

In teleosts, prolactin and cortisol are central hormones in freshwater (FW) and seawater (SW) adaptation, respectively (see reviews; Hirano, 1986; Bern and Madsen, 1992; McCormick, 1995). Recently, prolactin receptor (PRLR) gene was cloned in Nile tilapia (*Oreochromis niloticus*) (Sandra *et al.*, 1995) and cortisol receptor (CR) gene in rainbow trout (*Oncorhynchus mykiss*) and Mozambique tilapia (*O. mossambicus*) (Ducouret *et al.*, 1995; Tagawa *et al.*, 1997). Osmoregulatory surfaces such as the gills, kidney and intestine are the target organs of prolactin and cortisol as indicated by hormone binding assay in several teleost species (for prolactin, Edery *et al.*, 1984; Dauder *et al.*, 1990; Auperin *et al.*, 1994a, 1995; for cortisol, Sandor *et al.*, 1984; Chakraborti *et al.*, 1987; Weisbart *et al.*, 1987; Maule and Shreck, 1990; Shrimpton and Randall, 1994; Shrimpton *et al.*, 1995). In

agreement with the binding studies, the gills, kidney and intestine exhibit significant expression of PRLR and CR mRNAs (Ducouret *et al.*, 1995; Sandra *et al.*, 1995; Tagawa *et al.*, 1997; Uchida *et al.*, 1997).

Chloride cells or mitochondrion-rich (MR) cells in the gills and opercular membrane are the sites of ion transport particularly in fish in SW (Foskett and Scheffey, 1982; Zadunaisky, 1984; McCormick, 1995; Marshall, 1995). In the embryo and larva of euryhaline Mozambique tilapia, rich populations of chloride cells are found in the yolk-sac membranes prior to the development of the gills and opercular membrane (Ayson *et al.*, 1994a; Shiraishi *et al.*, 1997). When fish were transferred from FW to SW, there is a marked increase in the activity of chloride cells (Pisam, 1981; Foskett *et al.*, 1981, 1983; Pisam and Rambourg, 1991; Kültz *et al.*, 1992; Yoshikawa *et al.*, 1993; Ayson *et al.*, 1994a; Shiraishi *et al.*, 1997). Similar morphological changes in chloride cells have been seen in Mozambique tilapia after treatment of FW fish with cortisol (Foskett *et al.*, 1981; McCormick, 1990; Ayson *et al.*, 1995). On the other hand, transfer of the fish from SW to FW, or prolactin treatment of the fish in SW elicited de-differentiation of chloride cells (Herndon *et al.*, 1991; Pisam *et al.*, 1993).

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Little information is available on the endocrine control of hydromineral balance during early-life stages of fishes. In Mozambique tilapia, the beginning of embryonic synthesis of two forms of prolactins (PRL₁₈₈ and PRL₁₇₇) was identified at the time of hatching (Ayson *et al.*, 1994b) and cortisol synthesis several days after hatching (Hwang *et al.*, 1992; Hwang and Wu, 1993; Ayson *et al.*, 1995). On the other hand, tilapia eggs are known to contain maternal cortisol in the yolk (Hwang *et al.*, 1992; Hwang and Wu, 1993; Ayson *et al.*, 1995). Tagawa *et al.* (1997) demonstrated CR gene expression in the whole eggs of Mozambique tilapia just after fertilization. These results suggest possible roles for prolactin and cortisol during early development, possibly in the functional differentiation of chloride cells. We have recently established competitive reverse transcription-polymerase chain reaction (cRT-PCR) assay, which enabled us to quantify the relative levels of PRLR and CR mRNAs simultaneously in small amounts of tissue such as developing tilapia embryos. The present study describes the presence of PRLR and CR mRNAs in tilapia embryos and yolk-sac membranes, and their developmental changes in relation to environmental salinity.

MATERIALS AND METHODS

Fish

Mature Mozambique tilapia (*O. mossambicus*) were collected from a pond in northern Okinawa Island, Japan, and were maintained in tanks with re-circulating FW (Tokyo tap water) or natural SW (Cl⁻: 560 mM) at 25°C at the Ocean Research Institute, University of Tokyo. Fish were fed on artificial tilapia pellets ("Tilapia 41M", Shikoku Kumiai Shiryō, Tokushima, Japan) once a day. Male tilapia, weighing 30 to 40g, were used for cloning, and larger fish (100 to 150g) for quantification of PRLR and CR mRNAs. They were sacrificed after anesthesia with 0.1% 2-phenoxyethanol. Gills were immediately dissected, frozen in liquid nitrogen, and kept at -80°C until use.

Mature fish for breeding were kept in 200-l tanks, and mating behavior was checked hourly. The eggs just after fertilization were obtained from the mouth of brooding females. Otherwise, eggs were taken from the female 2 days after fertilization to minimize disturbance of the egg. Eggs were collected by keeping the mouth of the female open and flushing with water. Half of the eggs were transferred directly to natural SW 2 days after fertilization and the other half were maintained in FW. Two to three hundred eggs were incubated in each 10-l plastic tank at 25°C with aeration. The day of hatching was designated as day 0. Transfer of the egg to SW did not affect normal development of the embryos, and no mortality was seen after the transfer.

For examination of the developmental changes in the receptor mRNA, 10-30 embryos were collected on days -5 (fertilization), -3 (appearance of yolk-sac membrane), 0 (hatching) and 3 (early stages of gill development). At days -3, 0 and 3, yolk-sac membrane and embryonic or larval body were isolated. The embryo or larva was removed from the yolk sac, and the yolk-sac membrane was carefully peeled off the yolk in Ringer's solution (122 mM Na⁺, 124.6 mM Cl⁻, 3 mM K⁺, 1.25 mM Mg²⁺, 1 mM Ca²⁺, 1.25 mM SO₄²⁻, 0.4 mM PO₄³⁻, 2 mM CO₃²⁻, pH 7.4). They were frozen in liquid nitrogen and stored at -80°C.

RNA preparation

Total RNA was extracted from the frozen tissues by the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987). In the extraction from the yolk-sac membrane and the isolated

body, Ethachinmate (Nippon Gene, Tokyo), the carrier, was used to increase recovery rate of RNA. Concentrations of total RNA from the gills of adult fish and from whole embryos were quantified by spectrometric optical density at 260 nm.

Cloning

To establish a homologous cRT-PCR, a partial sequence of PRLR cDNA was first obtained. One µg of total RNA obtained from the gill of adult tilapia reared in FW was used to generate single-strand cDNA by SuperscriptTM reverse transcriptase (GIBCO BRL, Gaithersburg, MA) and oligo d(T)₁₂₋₁₈ as a primer. The cDNA was amplified by PCR using Takara Ex Taq polymerase (Takara Shuzo Co., Ltd., Tokyo), and 0.25 µM of each primers as follows: 5'-AATTCTGGATGTTGGC-GATAACC-3' and 5'-CATAGCTTCCATGACCAGAGTCAC-3'. These primers were designed based on the sequence of PRLR cDNA of Nile tilapia (*O. niloticus*) (Sandra *et al.*, 1995) and mouse (Moor and Oka, 1993). The PCR was performed in triplicate.

The PCR products were fractionated by agarose gel electrophoresis, and subcloned into pT7Blue(R) T-vector (Novagen, Madison, WI). This plasmid was called pT7_{PR} in this study. Nucleotide sequences of the insert was determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977), using the Thermo sequenase core sequencing kit (Amersham, Buckinghamshire) and the automated laser fluorescence sequencer (SQ-5500, Hitachi Electronics Engineering Co., Ltd., Tokyo).

Construction of competitor plasmids for cRT-PCR

The plasmid pT7_{PR} was digested with SacI and BamHI sites, located at position 692 and 896 respectively, to delete a 204-bp-long fragment. Blunting and ligation followed to obtain the competitor plasmid (pT7_{cPR}).

Tilapia cDNA regions of CR and β-actin (BA) as the control were amplified by PCR using primers as follows: for CR, 5'-TTTCGGTA-ATTGGTTGCTGATGAT-3' and 5'-AGTGCTCCTGGCTGTT(C,T)CT-(A,C,G,T)AGT-3' (Tagawa *et al.*, 1997); for BA, 5'-CTACGAGC-TGCCTGACGGACA-3' and 5'-CACATGTGCTGGAAGGTGGACA-3'. We referred to medaka (*Oryzias latipes*) and striped bass (*Morone saxatilis*) sequences for design of BA primers. PCR products were subcloned into pT7Blue(R) T-vector (pT7_{CR} and pT7_{BA}), and the sequences were confirmed as described above. Using these plasmids, inverse PCR was performed to generate deletion mutants of each tilapia DNA using primers designed in juxtaposition to each other. Resulting amplification of the entire vector and insert produced 70- and 58-bp deletions, respectively. The primers were as follows: for CR, 5'-AGCAGTGTTTGATGAGATTTCGC-3' and 5'-AGGTACTCATC-GTGGGAAACCTG-3'; for BA, 5'ATGCAGAAGGAGATCACAGCCC-3' and 5'-GATGTCAACGGTCGCACTTCATG-3'. The amplicons were subjected to blunting and ligation, and named pT7_{cCR} and pT7_{cBA}.

Synthesis of competitor RNA (cRNA)

Digestion of pT7_{cPR}, pT7_{cCR} and pT7_{cBA} with appropriate restriction enzymes resulted in linearized DNA fragments. Using these fragments as templates, sense-strand cRNAs were synthesized by T7 RNA polymerase (Stratagene, La Jolla, CA). The template DNA was subsequently removed by adding RNase-free DNase. The concentration of cRNA was quantified by absorbance at 260 nm. After adequate dilution, cRNA was stored at -80°C until use.

cRT-PCR

Our method of cRT-PCR was based on the procedure by Matsuda and Mori (1997) with some modification. The mixture of total RNA and adequate concentrations of the three cRNAs (for BA, PRLR and CR) were subjected to RT-PCR, using BcaBESTTM RNA PCR kit (Takara, Tokyo) and specific antisense primers. Reverse transcription was performed according to the recommended protocol of the kit with modification. After incubation at 55°C for 5 min, BcaBEST Polymerase was added to the reaction mixture. The temperature was then

maintained for 5 min prior to incubation at 65°C for 30 min. Subsequently, PCR was performed for 35 cycles (denaturation at 95°C for 30 sec, annealing at 59°C for 1 min, and extension at 72°C for 1 min) with 9.25 kBq of [α -³²P]-dCTP (Amersham, UK) and 0.25 μ M of each primers (see Fig. 1). After PCR, the products were separated by electrophoresis on 3.5% (for BA and PRLR) or 8% (for CR) acrylamide gel. Figure 1 schematically shows the amplified region of target RNAs and cRNAs. Radioactive incorporation of the PCR products was quantified on a Bioimage analyzer (Fuji BAS 2500; Fuji Film, Tokyo). The value of intensity of each DNA band was divided by the number of deoxycytidine residues contained in cRNA and the target RNA (BA, 76 and 98; PRLR, 154 and 202; CR, 28 and 44, respectively).

To examine the relationship between the initial ratio of target/competitor and the PCR product ratio of target/competitor, cRT-PCR was performed with fixed amount of target RNA and varying amount of cRNA. Subsequently, the logarithms of the PCR product ratio of target/competitor was plotted against the logarithms of the initial ratio of target/competitor RNA (Fig. 2). The results indicate that the initial ratio was maintained after cRT-PCR.

Statistical analysis

Statistical significance of differences between means was assessed with Student's *t*-test.

RESULTS

To establish a homologous cRT-PCR in the Mozambique tilapia (*O. mossambicus*), partial cloning of PRLR cDNA in

the gill was performed, and the nucleotide sequence consisting of 1222 bases has been registered in GenBank (accession number AF080247). The sequence exhibits a high homology (97.8%) with putative extracellular and transmembrane domains of PRLR of the Nile tilapia (*O. niloticus*).

The expression of PRLR and cortisol receptor (CR) mRNAs was quantified simultaneously using a newly developed cRT-PCR assay. Fig. 3 shows the effect of environmental salinity on the ratio of PRLR and CR mRNAs relative to BA mRNA in the gills of mature tilapia. In fish reared in FW, the expression of PRLR was significantly higher than that in SW-acclimated fish ($p < 0.05$). Conversely, the environmental salinity did not significantly affect gill CR mRNA content.

In the embryo just after fertilization (day -5), PRLR and CR mRNAs were already detected (Fig. 4). The PRLR mRNA levels relative to total RNA increased gradually during development, and became significantly greater in the embryo maintained in FW compared to those transferred to SW at hatching (day 0). Interestingly, CR mRNA levels were highest just after fertilization (day -5), decreased markedly toward hatching, and increased slightly thereafter. There was no significant difference between the embryos kept in FW and those transferred to SW.

To determine the major sites of the receptor mRNA ex-

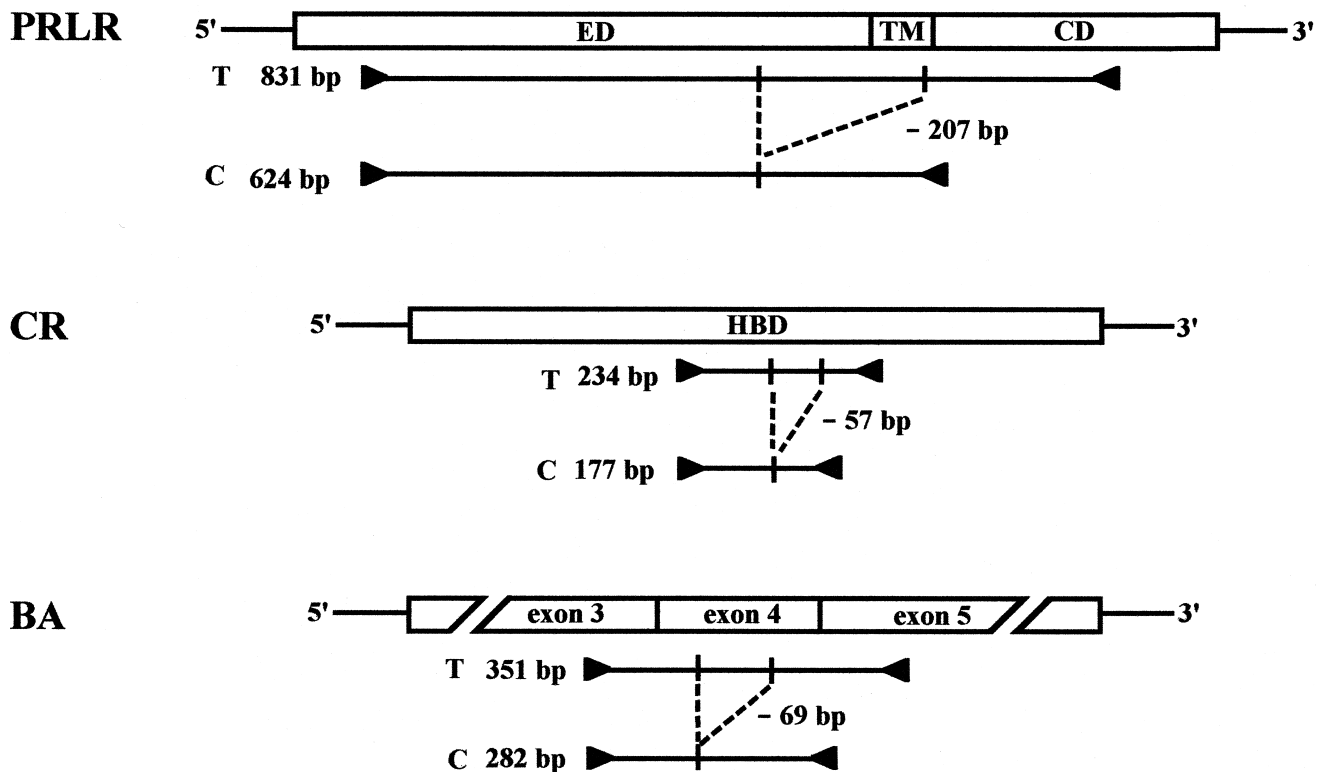


Fig. 1. Schematics of amplified regions from the target RNA (T) and competitor RNA (cRNA) (C) by competitive RT-PCR for prolactin receptor (PRLR), cortisol receptor (CR) and b-actin (BA). The depleted region in cRNA is indicated by broken lines. The sequences of primers (arrow-heads) are as follows: for PRLR, 5'-CAGAGATCAAATGCCGTTCTCC-3' and 5'-CCTCATAGTTAGACGTGGTTGG-3'; for CR, 5'-AATGAGGAGAGGATGAAGCTGC-3' and 5'-GTTCTGCTCACGCTTCACAATG-3'; for BA, 5'-CTACGAGCTGGCCTGACGGACA-3' and 5'-CACATGTGCTGGAAGGTGGACA-3'. Abbreviations: EM, extracellular domain; TM, transmembrane domain; CD, cytoplasmic domain; HBD, hormone-binding domain.

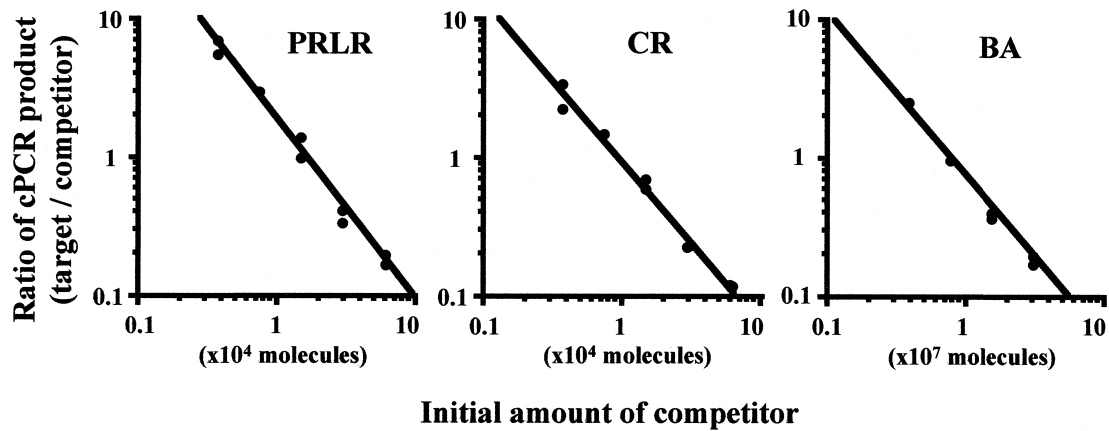


Fig. 2. Relationship between the initial ratio of target/competitor RNA and the PCR product ratio of target/competitor after competitive RT-PCR for prolactin receptor (PRLR), cortisol receptor (CR) and β -actin (BA).

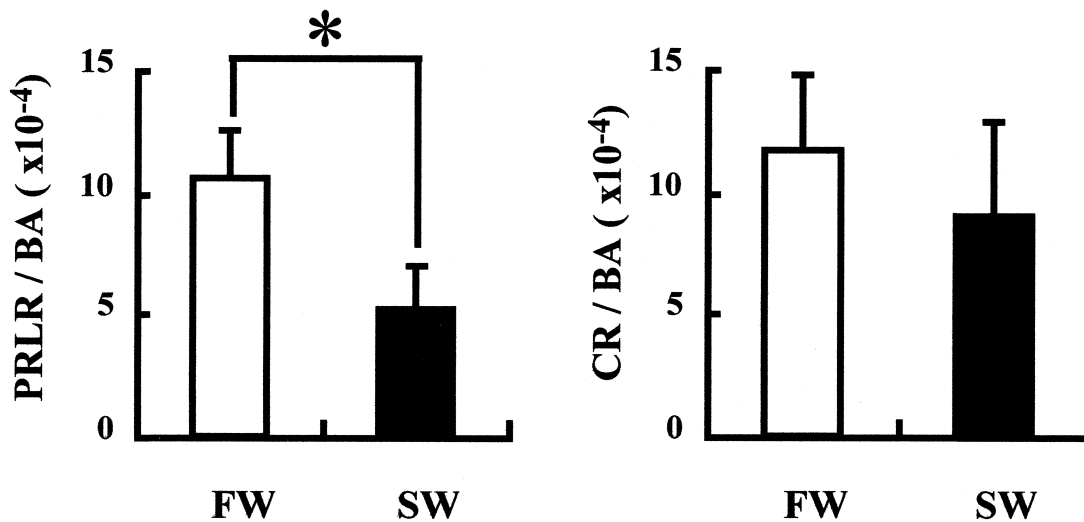


Fig. 3. Effect of environmental salinity on the ratio of PRLR mRNA and CR mRNAs to BA mRNA in the gills of mature tilapia. Vertical bars indicate S.E.M. (n = 4). * $p < 0.05$.

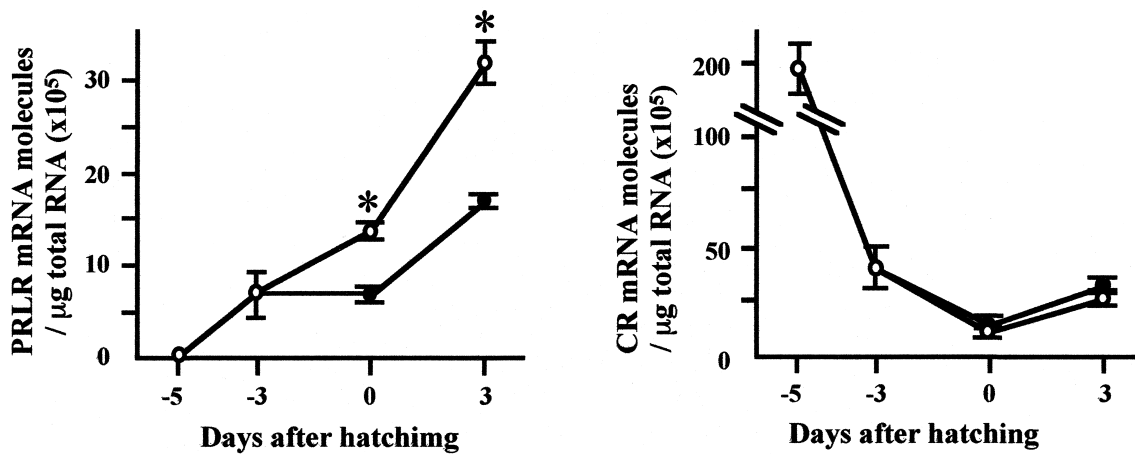


Fig. 4. Changes in the PRLR mRNA and CR mRNA in whole eggs during development. Half of the eggs were maintained in FW (○) and the other half were transferred to SW 3 days before hatching (●). Data are expressed as concentrations of the receptor mRNAs in 1μ g of total RNA. Eggs from 4 brooding females were used for the quantification. * $p < 0.05$.

pression, cRT-PCR was also performed on the embryos or larvae and on the yolk-sac membrane separated from the yolk. As the sample size was too small to estimate total RNA concentrations, the ratios of PRLR/BA and CR/BA were adopted. As shown in Fig. 5, both PRLR and CR mRNAs were detected in the embryonic body and in the yolk-sac membrane as early as 3 days before hatching. Changes in salinity affected the ratio of PRLR/BA in the isolated body but not in the yolk-sac membrane. On day 3, the ratio of PRLR/BA in the larval body isolated from FW-reared larva was significantly greater than that in SW larvae. On the other hand, there was no difference in the level of PRLR mRNA in the yolk-sac membrane between the two groups. The CR mRNA concentration

in the embryonic and larval body and in the yolk-sac membrane showed no detectable change following transfer from FW to SW.

DISCUSSION

In the present study, concentrations of PRLR and CR mRNAs were quantified by cRT-PCR assay. This method is useful for quantifying levels of mRNA in small samples such as tilapia embryos and yolk-sac membrane, tissues which express low levels of mRNA. The tilapia pituitary is known to produce and secrete two PRLs, PRL₁₈₈ and PRL₁₇₇ or PRL_I and PRL_{II} (Specker *et al.*, 1985; Yamaguchi *et al.*, 1988;

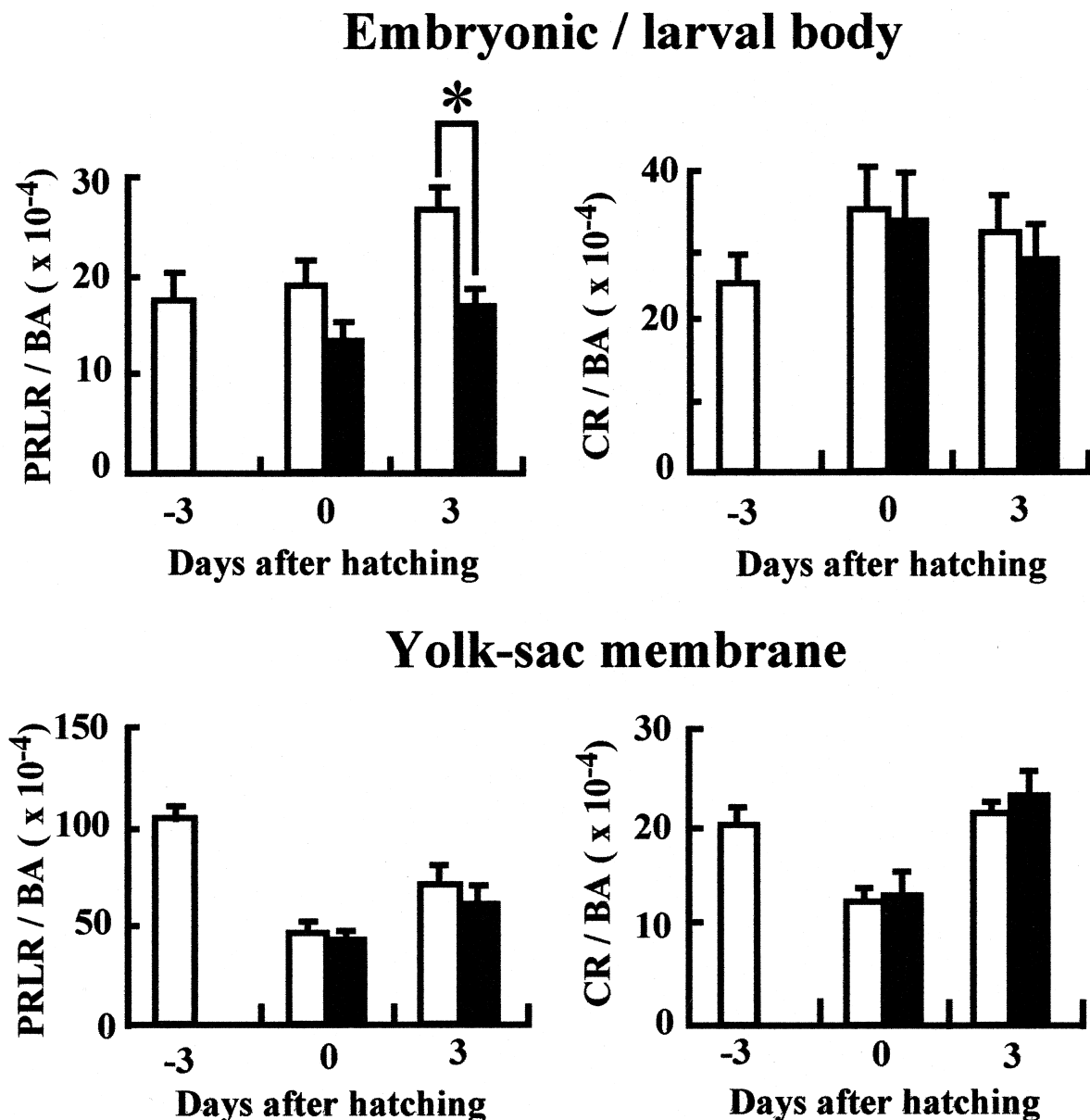


Fig. 5. Effect of environmental salinity on the ratio of PRLR mRNA and CR mRNAs to BA mRNA in the embryonic and larval body and in the yolk-sac membrane. Half of the eggs were maintained in FW (clear column) and the other half were transferred to SW 3 days before hatching (solid column). Vertical bars indicate S.E.M. (n = 4). *p<0.05.

Rentier-Delrue *et al.*, 1989). Auprin *et al.* (1994a) characterized a single PRLR in Nile tilapia (*O. niloticus*), which binds both PRLs. As shown in the present study, levels of PRLR mRNA relative to BA mRNA (PRLR/BA) in the gills of Mozambique tilapia (*O. mossambicus*) were significantly higher in fish kept in FW than in those in SW. This is consistent with the change in PRLR mRNA in the gills following transfer of Nile tilapia to blackish water (20‰), as measured by Northern-blot analysis (Sandra *et al.*, 1995). The expression of PRLR mRNA in the gills seems to be up-regulated by the higher levels of circulating PRLs present in FW than in SW or in blackish water (Ayson *et al.*, 1993; Auperin *et al.*, 1994b). According to Auperin *et al.* (1995), however, specific binding of PRLs to the gill membrane of Nile tilapia was significantly higher in the fish kept in blackish water than in FW. This suggests that the receptor protein is down-regulated at high concentrations of circulating PRLs in FW. Genty *et al.* (1994) reported that internalization of the cell surface PRLR was enhanced in the presence of PRL, leading to apparent down-regulation of the receptor. In mammals, the complexity of up- and down-regulation of PRLR gene expression has been well documented, depending on target organs, hormone concentrations and duration of the exposure to the hormone (Kelly *et al.*, 1991; Prunet and Auperin, 1995). Further studies are needed to elucidate the regulatory mechanisms of PRLR at transcript and protein levels in teleosts.

Ayson *et al.* (1994b) reported significantly higher expression of PRL₁₈₈ mRNA in the pituitary of tilapia (*O. mossambicus*) larva reared in FW than those in SW at the time of hatching, and in PRL₁₇₇ mRNA 2 days after hatching. In the present study, levels of PRLR mRNA were also higher in the whole embryo and larva kept in FW as well as in the embryonic body separated from the yolk at the time of hatching and in the larval body 3 days after hatching, as compared with those in SW. These results clearly indicate the presence of regulatory mechanisms of the hormone production in the pituitary and possibly its release into circulation as well as the expression of the PRLR gene in early-life stages of tilapia.

In higher vertebrates, functional versatility of PRL and adrenal corticoids extends to growth-promoting effects during early development. In cultured rat embryos, Karabulut and Pratten (1998) demonstrated the potency of PRL as a growth-promoting molecule during embryogenesis. On the other hand, Cole *et al.* (1995) showed that targeted disruption of the glucocorticoid receptor (GR) gene, causing lethal abnormalities of the lung and adrenal gland in the mice. Significant expression of PRLR and CR mRNAs in tilapia eggs as early as just after fertilization suggests important roles for these hormones during embryogenesis.

Hwang and Wu (1989) reported more advanced development of the kidney in Mozambique tilapia larva hatched in FW than in SW. According to Li *et al.* (1995), numerous functional chloride cells or MR cells are present in the newly developed gills of tilapia larvae as early as 3 days after hatching in FW. On the other hand, we have reported that chloride cells in the yolk-sac membrane are more developed in tilapia em-

bryos and larvae in SW than those in FW (Ayson *et al.*, 1994a; Shiraishi *et al.*, 1997). Although chloride cells are well recognized as the site of ion extrusion in the fish in SW, their function in FW is still disputed (Goss *et al.*, 1995; Marshall, 1995). Thus, the greater expression of PRLR gene in whole larvae and in the isolated embryonic or larval body in FW implies a role for prolactin in differentiation of osmoregulatory surfaces including FW-type chloride cells. However, there was no significant change in the ratio of PRLR/BA in the yolk-sac membrane after transfer to SW, suggesting that prolactin may be involved in the maintenance and/or function of the yolk-sac membrane, in addition to differentiation of chloride cells.

Several steroid and thyroid hormones are present in the unfertilized egg of various teleost species (see a review by Tagawa, 1996). Recently, Green and Chen (1997) reported mRNA of insulin-like growth factors (IGFs) in unfertilized eggs of rainbow trout. Peptide hormones, including IGFs and prolactin, may also be among the maternal hormones/factors utilized by the fish embryo. Indeed, the present study demonstrates significant expression of PRLR and CR genes in the newly developed yolk-sac membrane (day -3). The organ culture of the yolk-sac membrane of the tilapia embryo 1 day before hatching resulted in a decrease in chloride cell density, and the decrease was countered by adding cortisol to the culture medium (Ayson *et al.*, 1995). Thus, cortisol is likely to play an important role in the differentiation and/or proliferation of chloride cells. Interestingly, the highest concentration of CR mRNA was found just after fertilization in this study. The result suggests that the maternal cortisol and the receptor are utilized in the developing embryo before the commencement of its own production.

Environmental salinity, however, did not affect the CR mRNA expression in any tissues at all stages examined in the present study. Although the level of CR expression is under control of various factors including steroids and neurotransmitters, cortisol itself seems to be the most important regulator (Bamberger *et al.*, 1996). In the gill of coho salmon (*O. kisutch*), cortisol treatment caused the down-regulation of the binding site (Maule and Schreck, 1991; Shrimpton and Randall, 1994). In Mozambique tilapia, however, there was no difference in the plasma cortisol level between fish in FW and those in SW, although cortisol production by the interrenal was greater in SW fish (Balm *et al.*, 1995). Similarly, cortisol contents in the whole embryo and larva of Mozambique tilapia were not affected by environmental salinity (Hwang and Wu, 1993; Ayson *et al.*, 1995). In chum salmon (*O. keta*) fry, Uchida *et al.* (1997) reported that CR gene expression, examined by in situ hybridization and immunostaining with anti-CR serum, was found in two types of chloride cells in the gill filaments and lamellae. However, there was no difference in plasma cortisol nor in the expression of CR gene. Cortisol sensitivity could be regulated by mRNA stability, translatability, and turnover of the receptor protein. Heat shock proteins and transcriptional factors may also modify the hormone-binding affinity to the receptor and the transcriptional activities of the target gene (Bamberger *et al.*, 1996). In fact, fluctuations of

the both were observed in the gills of a goby, *Gillichthys mirabilis*, following osmotic shock (Kültz, 1996).

The present study demonstrates the expression of PRLR and CR in the yolk-sac membrane and embryonic body of tilapia, suggesting that prolactin and cortisol may be involved in growth and development, and/or hydromineral balance of tilapia embryos, possibly by affecting functional differentiation of chloride cells. However, direct effect of the hormones or specific localization of their receptors on chloride cells or other cell types have not been demonstrated yet. Further studies by means of in situ hybridization will provide useful information on the sites of expression of PRLR and CR genes on specific cell types in the developing embryos

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