Is the primitive regulation of pituitary prolactin ($tPRL_{177}$ and $tPRL_{188}$) secretion and gene expression in the euryhaline tilapia (*Oreochromis mossambicus*) hypothalamic or environmental?

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Abstract

We examined the effects of environmental salinity on circulating levels of the two prolactins ($tPRL_{177}$ and $tPRL_{188}$) and levels of pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA in the euryhaline tilapia, *Oreochromis mossambicus*. Fish were sham-operated or hypophysectomized and the rostral pars distalis (RPD) autotransplanted onto the optic nerve. Following post-operative recovery in ¼ seawater, tilapia were transferred to fresh water (FW), ¼ seawater (SW) or SW. Serum $tPRL_{177}$ and $tPRL_{188}$ levels in sham-operated and RPD-autotransplanted fish were highest in FW and decreased as salinity was increased. $tPRL_{177}$ and $tPRL_{188}$ mRNA levels in RPD implants as well as in pituitaries from the sham-operated fish were also highest in FW and decreased with increasing salinity. Serum osmolality increased with salinity, with the highest levels occurring in the seawater groups. We conclude that some plasma factor (probably plasma osmolality), in the absence of hypothalamic innervation, exerts a direct regulatory action on prolactin release and gene expression in the pituitary of *O. mossambicus*. This regulation is in accord with the actions of the two prolactins in the freshwater osmoregulation of the tilapia.

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Introduction

Early studies employing the use of ectopic pituitary autotransplants in mammals (Adler 1986) and in lower vertebrates, specifically teleosts (see Ball 1981, Nishioka *et al.* 1988), established that pituitary prolactin (PRL) secretion is under inhibitory control by the hypothalamus. In mammals, this conclusion was based on the fact that the ectopic transplantation of the anterior pituitary to the kidney capsule results in elevated levels of circulating PRL (Adler 1986). In teleosts, this finding has been based on less direct observations from *in vitro* studies (see Nishioka *et al.* 1988) and studies involving the cytological examination of the active ectopic anterior pituitary tissues (see Olivereau 1969, Ball *et al.* 1972, Ball 1981); to date, no direct measurements of circulating PRL levels have been made.

In the tilapia (*Oreochromis* spp.), two forms of prolactin have been identified, one of which contains 177 amino acid residues ($tPRL_{177}$), and the other, 188 amino acid residues ($tPRL_{188}$) (Specker *et al.* 1985, Yamaguchi *et al.* 1988). The amino acid sequence identity of the two prolactins is only 69%, with each form being encoded by separate genes (Yamaguchi *et al.* 1988, Rentier-Delrue *et al.* 1989). Given these differences, studies have aimed at determining their unique physiological functions in ion and water balance (Specker *et al.* 1985, Young *et al.* 1988, Specker *et al.* 1989, Auperin *et al.* 1994, 1995, Flik *et al.* 1994, Sakamoto *et al.* 1997), growth (Shepherd *et al.* 1997*b*), reproduction (Rubin & Specker 1992, Oshima *et al.* 1996) and pigmentation (Kitta *et al.* 1993, Oshima *et al.* 1996). Both forms of PRL are colocalized within the same cells of the rostral pars distalis (RPD) of the pituitary (Nishioka *et al.* 1993, Specker *et al.* 1993), but when the ratio of the two prolactins ($tPRL_{188}$:$tPRL_{177}$) is examined, it becomes evident that they are differentially regulated during development (Ayson *et al.* 1994), and by changes in environmental salinity (Borski *et al.* 1992, Auperin *et al.* 1994, Yada *et al.* 1994, Yoshikawa-Ebesu *et al.* 1995) and nutrition (Vijayan *et al.* 1996, Shepherd *et al.* 1997*a*). The

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role of the hypothalamus, if any, in the differential regulation of the two PRLs in tilapia is not understood.

We and others have been interested in examining the *in vivo* regulation of pituitary PRL secretion and induction of mRNA encoding for tilapia PRLs to clarify the degree to which environmental salinity affects plasma osmolality and, hence, PRL levels in tilapia (Nicoll *et al.* 1981, Auperin *et al.* 1994, Yada *et al.* 1994). Pioneering studies in various teleosts including tilapia identified an inverse relation between environmental salinity and prolactin cell activity *in vivo* (Dharmamba & Nishioka 1968, Olivereau 1969, Ball 1969*a,b*) and between medium osmolality and PRL release *in vitro* (Sage 1965, 1968, Ingleton *et al.* 1973, Nagahama *et al.* 1974, Zambrano *et al.* 1974). These findings would seem to indicate that the PRL cell activity of these teleosts reflects changes in extracellular osmolality during adaptation to different salinities.

Studies conducted in our own and others' laboratories, measuring plasma PRL and pituitary PRL mRNA levels, argue that the PRL cell activity of tilapia adapted to fresh water (FW) is higher than that of tilapia adapted to seawater (SW) (Nishioka *et al.* 1993, Auperin *et al.* 1994, Yada *et al.* 1994, Shepherd *et al.* 1997*a*). Additionally, we have found good correlations among environmental salinity, plasma osmolality and PRL cell activity in the tilapia (Yada *et al.* 1994). On the other hand, Wendelaar Bonga *et al.* (1980, 1981), using cytological (morphometric analysis) methods, reported that PRL cell activity in tilapia adapted to an hyperosmotic environment was greater than that seen in tilapia adapted to fresh water. Further, they have not found consistent correlations between plasma osmolality and PRL cell activity in tilapia (Wendelaar Bonga *et al.* 1980, 1981, 1985, 1988). Based on their findings, they have questioned the physiological significance of the *in vitro* hypo-osmotic activation of the tilapia PRL cell demonstrated in earlier studies (e.g. Zambrano *et al.* 1974, Nagahama *et al.* 1975, Wigham *et al.* 1977, Grau *et al.* 1981), and thus maintain that PRL cell activity is controlled principally by the hypothalamus, rather than by any direct effects of plasma osmolality. However, Bern (1980), Loretz & Bern (1982) and Grau *et al.* (1994) in a series of reviews suggest that alteration in ambient salinity and associated plasma osmolality may be the primitive/primary factor regulating pituitary PRL secretion and gene expression in teleosts. This suggests that the mechanisms of PRL cell control that are present *in vitro* may also be active *in vivo* in the tilapia during adaptation to different salinities. We propose that small changes in tissue fluid osmolality, following transfer to different salinities, may be the principal regulator of PRL release in the tilapia, and that the aforementioned differences may relate to a variety of methodologies used to assess PRL cell activity.

Against this background, we have undertaken studies aimed at determining whether the release of pituitary PRL is a direct response to changes in extracellular osmolality *in vivo* when PRL cells are separated from the hypothalamus. The euryhaline tilapia, *Oreochromis mossambicus*, is the focus of our studies because its pituitary cells reflect changes in medium osmolality *in vitro* and in environmental salinity *in vivo*, they are easily accessed and because the arrangement of its pituitary facilitates study. The structure of the tilapia pituitary is advantageous for studying the regulation of PRL release and synthesis because the PRL cells are segregated into a nearly homogenous mass located in the rostral pars distalis and comprise greater than 95% of the cells in this region of the pituitary (see Dharmamba & Nishioka 1968, Nishioka *et al.* 1993). This region of the pituitary can be easily dissected to obtain a nearly homogenous population of PRL-secreting cells, devoid of growth hormone (GH)-secreting cells (Dharmamba & Nishioka 1968, Nishioka *et al.* 1993).

Here, we have used ectopic pituitary autotransplants of the rostral pars distalis to examine whether levels of the tilapia PRLs ($tPRL_{177}$ and $tPRL_{188}$) vary *in vivo* with changes in environmental salinity and plasma osmolality consistent with their role in FW osmoregulation. We report the effects of environmental salinity on circulating and pituitary levels (autotransplanted and *in situ*) of $tPRL_{177}$ and $tPRL_{188}$ as well as mRNA levels and blood osmolality.

Materials and Methods

Animals

Tilapia (*Oreochromis mossambicus*) were reared in circular 6000-liter tanks in FW and under natural photoperiod at the Hawaii Institute of Marine Biology, Kaneohe, Hawaii. Animals were fed Purina Trout Chow twice daily (ration was approximately 2% of body weight per day). Food was withheld 48 h prior to surgery. Water temperature was 25 ± 2 °C.

Experimental protocols

Study I Tilapia of both sexes (60–90 g) were anesthetized and hypophysectomized (Hx) by the transorbital procedure as described previously (Nishioka 1994). The RPD was dissected from the pituitary, which was removed intact, in 355 mosmolal tilapia Ringer (Wigham *et al.* 1977) and placed with forceps back into the hypothalamic area; the implant frequently attached itself to the optic tract. Sham-operated and Hx animals not receiving pituitary autotransplants were used as controls. The animals were tagged in the dorsal musculature with sequentially numbered T-tags (HallPrint T-tags, South Australia) for individual identification.

Following a two-week post-operative recovery in ¼ SW, animals in each group (sham, n=5; RPD, n=8) were sampled for plasma and hypophyseal tissues (initial groups) and the remaining animals were separated (sham, n=5–10; RPD, n=5–10 per salinity) and acclimated to different salinities in one of three 600-liter tanks containing FW, ¼

SW or SW (32 p.p.t.). Animals transferred from ¼ SW to ¼ SW (to assess the effect of handling stress) served as controls for salinity transfer. The animals held in ¼ SW tanks were maintained in filtered, recirculated water, whereas fish in the FW (municipal water) and SW (sand-filtered) tanks were subject to flow-through conditions. During this period, the animals were treated with antibiotics (Maracyn-I and -II, Mardel Laboratories, Glendale Heights, IL, USA) according to the manufacturer's recommendations. The animals were fed twice daily to satiation (Purina Trout Chow) and all tanks were siphoned daily; half of the water in the ¼ SW tanks was also replaced at this time. The animals were held in these tanks for a period of 3 months and growth was monitored two weeks after the initial salinity acclimation and every 4 weeks thereafter. Mortality did not exceed 10% for the sham-operated and RPD-autotransplanted groups.

The animals were held for a period of three months and then sampled. At the time of sampling, the animals were anesthetized and weight and length were determined, blood was collected and animals were killed as described elsewhere (Sakamoto *et al.* 1997). The Hx controls did not survive the duration of the study. After centrifugation, the serum was stored at −80 °C. Pituitaries and RPD fragments were removed, frozen in liquid nitrogen, and stored at −80 °C for subsequent mRNA analyses.

Study II Tilapia of both sexes (60–90 g) were Hx, sham-operated or received autotransplants of the RPD as described above. Following surgery, the animals were transferred to oval 60-liter tanks supplied with recirculated, filtered ¼ SW as described above. Following post-operative recovery (10 days), animals (n=6 per group) were sampled for tissues (serum, pituitaries and RPD fragments). Pituitary tissues were fixed in 4% paraformaldehyde for 24 h at 4 °C for ancillary *in situ* hybridization studies (Nishioka *et al.* 1993). The remaining animals from each of the three groups (n=9 per group) were acclimated, over the period of 1 week, to different salinities (FW, ¼ SW or SW) as before. Animals transferred from ¼ SW to ¼ SW (to assess the effect of handling stress) served as controls for salinity transfer. Fourteen days after transfer, the animals were sampled as described above.

Radioimmunoassay and serum osmolarity

Serum levels of $tPRL_{177}$ and $tPRL_{188}$ were quantified using the RIA procedures of Ayson *et al.* (1993) as modified by Yada *et al.* (1994). Serum osmolality was determined from replicate 10-µl serum samples using a vapor pressure osmometer (Wescor 5100C, Logan, Utah, USA).

Northern blot analyses for $tPRL_{177}$ and $tPRL_{188}$ mRNA

Total RNA was extracted from pituitaries using the single-step acid guanidinium–phenol–chloroform extraction (Chomczynski & Sacchi 1987). Two pituitaries or RPD were pooled to extract total RNA, and RNA was extracted from the different groups at the same time to avoid procedure variability in extraction among the experimental groups. Extracted RNA samples were dissolved in 10 µl Ultrapure water and stored at −80 °C.

Oligonucleotide probes (45 mers) for tilapia PRL_{177} and PRL_{188} corresponding to amino acid residues 90 to 104 (a region of major dissimilarity between the tilapia PRLs) of the PRL molecules of *O. niloticus* (Rentier-Delrue *et al.* 1989) and *O. mossambicus* (Yamaguchi *et al.* 1988, Nishioka *et al.* 1993) were labeled at the 3′-tail with [α-^{32}P]dATP using labeling kit N4020 (Amersham, Arlington Heights, IL, USA). Probe specificity was confirmed by Northern blot analysis and also by competition tests from *in situ* hybridization studies for PRL probes (Nishioka *et al.* 1993).

For Northern blot analyses, 5 µl (RPD) and 2·5 µl (intact pituitary) samples were used. Total RNA samples were electrophoresed through a 1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell, Keene, NH, USA) by capillary blotting (Shambrook *et al.* 1989). The RNA was covalently attached to the membrane by UV cross-linking. Following prehybridization, the membranes were hybridized sequentially with the oligonucleotide probes for $tPRL_{188}$ and $tPRL_{177}$ at 50 °C for 18 h according to the method of Shambrook *et al.* (1989). The membranes were washed in 0·5 × SSC/0·01% SDS at room temperature for 10 min and then washed twice for 1 h at 50 °C.

Intensity of hybridization signals was estimated with an Auto Image Analyzer (BasMac, Fuji Film, Tokyo, Japan). Autoradiography was then performed by exposing Hyperfilm-MP (Amersham) to the membranes at −80 °C with a Toshiba E-32 intensifying screen. After development, the hybridized probe was removed by soaking the membranes in 10 mM Tris (pH 8·0), 1 mM EDTA and 0·1% SDS at 65 °C for 2 h. The membranes were then hybridized with the oligonucleotide probe for $tPRL_{177}$. Relative abundance of PRL mRNAs was assessed as autoradiographic intensity per single pituitary or RPD. Serial dilutions of total RNA from pooled pituitaries demonstrated linearity between hybridization signals and serial dilutions (data not shown). Messenger RNA data are represented in optical density units per RPD or pituitary (sham-operated animals) and were not normalized, since variable amounts of neural tissue adhered to the RPD fragments, thus contributing to total extracted RNA. For this reason, we have not made any statistical comparisons of the PRL mRNAs between the sham-operated and RPD-autotransplanted groups.

Statistical analysis

Differences among groups were evaluated by analysis of variance (ANOVA) (Minitab, State College, PA, USA),

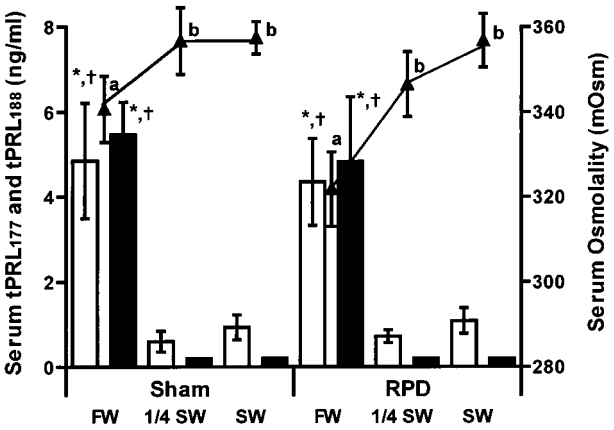

Figure 1 Effects of salinity transfer from ¼ SW to FW, ¼ SW or SW on circulating levels of $tPRL_{177}$ (solid bars) and $tPRL_{188}$ (open bars) in sham-operated and RPD-autotransplanted tilapia of study I. The salinity indicated represents the salinity in which the animals were sampled. Values for hormone levels are means ± S.E.M. and are expressed as ng/ml serum (n=5–10 per group). *P<0·001 compared with the respective ¼ SW group; †P<0·001 compared with the respective SW group. Values for serum osmolality (closed triangles) are means ± S.E.M. and are expressed in milliosmolals (n=5–10 per group). Groups with different letters are significantly (P<0·05) different from other values within the respective experimental groups (sham-operated or RPD-autotransplanted).

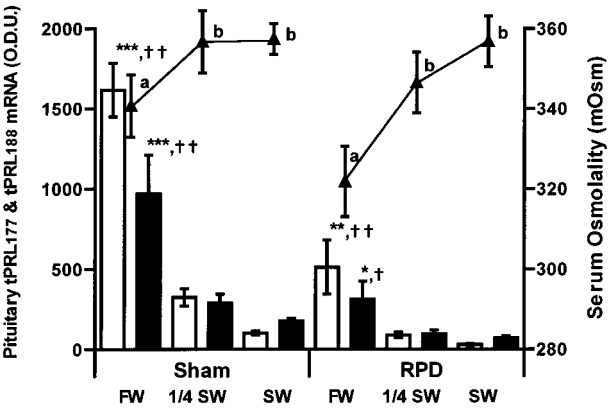

Figure 2 Effects of salinity transfer from ¼ SW to FW, ¼ SW or SW on $tPRL_{177}$ (solid bars) and $tPRL_{188}$ (open bars) mRNA levels in pituitary tissues from sham-operated and RPD-autotransplanted tilapia of study I. The salinity indicated represents the salinity in which the animals were sampled. Values are means ± S.E.M. and are expressed as arbitrary optical density units (O.D.U) (n=3–6 per group). *P<0·05, **P<0·005, ***P<0·001 compared with the respective ¼ SW group; †P<0·01, ††P<0·001 compared with the respective SW group. Values for serum osmolality (closed triangles) are means ± S.E.M., expressed in milliosmolals (n=5–10 per group), and are the same as shown in Fig. 1. Groups with different letters are significantly (P<0·05) different from other values within the respective experimental groups (sham-operated or RPD-autotransplanted).

followed by Fisher's least significant difference test (Fisher's protected least significant difference, FPLSD) for predetermined pairwise comparisons, unless stated otherwise. The mean square error value used to calculate the LSD was derived from one-way analysis of variance (ANOVA). One-tailed alternatives were used to obtain the upper critical value for use in the LSD test (Steele & Torrie 1980).

Results

Serum hormone levels and osmolality

Study I To evaluate the direct effects of environmental salinity on PRL regulation, we measured levels of circulating $tPRL_{177}$ and $tPRL_{188}$ in the blood and pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA in intact tilapia and tilapia bearing long-term (>3·0 months) ectopic RPD autotransplants. Over the course of the study, RPD-autotransplanted fish increased in weight, whereas the sham-operated animals grew predominantly in length (data not shown). Transfer from ¼ SW to ¼ SW resulted in no significant (P>0·05) change in serum levels of $tPRL_{177}$ or $tPRL_{188}$, whether measured in the sham-operated or RPD-autotransplanted groups (data not shown); this suggests that there was no effect of handling on prolactin levels. Serum levels of $tPRL_{177}$ and $tPRL_{188}$ were significantly (P<0·001, FPLSD) higher in FW-adapted groups (RPD and sham) than in fish adapted to either ¼ SW or SW (Fig. 1); however, the mean ratios ($tPRL_{188}$:$tPRL_{177}$) of the two prolactins increased (not significantly) from 5·6 ± 1·3 in ¼ SW to 7·6 ± 0·5 in SW in the sham-operated animals and from 3·6 ± 0·8 in ¼ SW to 5·4 ± 1·5 in SW in the RPD-autotransplanted animals. The ratio of the two prolactins in the FW-adapted sham-operated and RPD-autotransplanted groups was not significantly (P>0·05) different (0·9 ± 0·15 and 1·4 ± 0·6 respectively). Serum osmolality significantly (P<0·05) decreased with salinity, with lower levels occurring in the FW sham-operated and RPD-autotransplanted groups. Individual statistical comparisons for serum osmolality among the experimental groups are presented in Fig. 1.

Pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA levels

Study I Pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA levels were significantly higher in the FW-adapted, sham-operated (P<0·001, FPLSD) and RPD-autotransplanted (P<0·05) groups than in ¼ SW or SW (Fig. 2). Pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA levels were found at equally low levels in the ¼ SW- and SW-adapted sham-operated and RPD-autotransplanted groups (Fig. 2). Thus, individual pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA levels in the sham-operated and RPD-autotransplanted groups did not increase until salinity was reduced below ¼ SW (Fig. 2); however, the ratio ($tPRL_{188}$:$tPRL_{177}$) of the two PRL mRNAs in these groups was significantly (P<0·05) lower

in SW (RPD, 0·4 ± 0·04; sham, 0·7 ± 0·1) than in ¼ SW (RPD, 1·0 ± 0·1; sham, 1·0 ± 0·1). The ratios (see values below) of the two PRL mRNAs in the sham-operated and RPD-autotransplanted groups were significantly ($P<0·001$) higher in the FW than in corresponding SW groups; however, within the same salinity, these ratios were not significantly ($P>0·05$) different between the sham-operated (FW, 1·7 ± 0·1; ¼ SW, 1·1 ± 0·1; SW, 0·7 ± 0·13) and RPD-autotransplanted (FW, 1·6 ± 0·1; ¼ SW, 1·0 ± 0·1; SW, 0·4 ± 0·04) groups.

Study II This study was undertaken to determine whether RPD-autotransplanted and sham-operated tilapia respond similarly to changes in environmental salinity as seen in study I. Here we chose a shorter time-course because our first study demonstrated that the autotransplanted RPD functionally recovers within two weeks of surgery. Levels of $tPRL_{177}$, $tPRL_{188}$ and GH (ng/ml) in Hx controls for this study were 0·2 ± 0·01 (0·2), 0·7 ± 0·04 (0·7) and 0·2 ± 0·1 (0·2) respectively (values in parentheses are the minimum detectable levels for that hormone).

The regeneration of pituitary remnants following hypophysectomy, a possibility in our study, has been described for the killifish, *Fundulus heteroclitus* (Ball 1965). Four lines of evidence, however, argue against the presence of regenerated pituitary remnants in our study. First, no regenerated pituitary tissue in the area of the hypothalamus was observed in Hx controls upon post-mortem examination. Secondly, levels (ng/ml) of $tPRL_{177}$, $tPRL_{188}$ and GH in Hx controls of study II (see above) were not significantly different from the minimum detectable levels and were significantly ($P<0·05$, *t*-test) lower than levels seen in the corresponding sham-operated control group. Thirdly, the transfer of Hx animals to FW (studies I and II) resulted in death within 7 days. Fourthly, the aspirated pituitaries were checked microscopically for intactness, and damaged pituitaries were discarded. These results argue that the fish were indeed hypophysectomized.

Transfer from ¼ SW to ¼ SW (controls) resulted in no significant ($P>0·05$) change in serum levels of $tPRL_{177}$ or $tPRL_{188}$ in either sham-operated or RPD-autotransplanted groups (data not shown), suggesting that there were no effects of handling on circulating prolactin levels in the RPD-autotransplanted and sham-operated groups. Serum levels of $tPRL_{177}$ and $tPRL_{188}$ in RPD-autotransplanted fish were significantly ($P<0·001$, FPLSD) higher in FW than in ¼ SW or SW (Fig. 3). Serum levels of $tPRL_{177}$ in sham-operated fish were significantly ($P<0·005$, FPLSD) higher in FW than in ¼ SW or SW (Fig. 3). However, serum levels of $tPRL_{188}$ remained low in FW-adapted sham-operated animals and were not significantly ($P>0·05$) different (although mean levels were higher) from those in fish adapted to either ¼ SW or SW (Fig. 3). Although $tPRL_{188}$ levels in the FW-adapted, sham-operated group were low (unlike

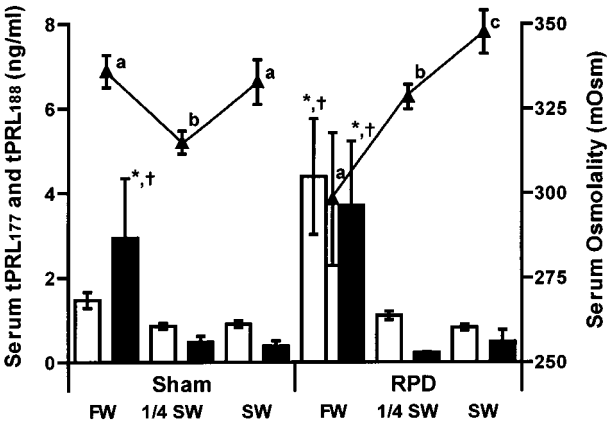

Figure 3 Effects of salinity transfer from ¼ SW to FW, ¼ SW or SW on circulating levels of $tPRL_{177}$ (solid bars) and $tPRL_{188}$ (open bars) in sham-operated and RPD-autotransplanted tilapia of study II. The salinity indicated represents the salinity in which the animals were sampled. Values for hormone levels are means ± S.E.M. and are expressed as ng/ml serum (n=7–9 per group). *$P<0·005$ compared with the respective ¼ SW group; †$P<0·005$ compared with the respective SW group. Serum $tPRL_{188}$ levels in the FW-RPD group were significantly ($P<0·001$, FPLSD) higher than levels in the FW-sham group. Values for serum osmolality (closed triangles) are means ± S.E.M. and are expressed in milliosmolals (n=5–9 per group). Groups with different letters are significantly ($P<0·05$) different from other values within the respective experimental groups (sham-operated or RPD-autotransplanted).

in the RPD-autotransplanted group), the ratio of the two prolactins ($tPRL_{188}$:$tPRL_{177}$) in the FW-adapted, sham-operated (3·3 ± 0·95) and RPD-autotransplanted (4·5 ± 2·2) groups was not significantly different. There were no significant ($P>0·05$) differences between circulating levels of $tPRL_{177}$ or $tPRL_{188}$ in animals (sham-operated or RPD-autotransplanted) adapted to ¼ SW and SW (Fig. 3), although the mean ratio of the two prolactins in the sham-operated group increased from 2·6 ± 0·5 in ¼ SW to 3·6 ± 0·6 in SW (not significant), but decreased in the RPD-autotransplanted group (4·6 ± 2·2 in ¼ SW to 3·4 ± 0·5 in SW). Serum $tPRL_{188}$ levels in the FW-RPD group were significantly ($P<0·001$, FPLSD) higher than corresponding levels in the FW-sham group. In contrast, serum levels of $tPRL_{177}$ in the FW-RPD and FW-sham groups were not significantly ($P>0·05$) different (Fig. 3).

Serum osmolality (milliosmolals) in the control Hx animals transferred to ¼ SW from ¼ SW was significantly ($P<0·01$, FPLSD) higher than initial levels, indicating that there may be an effect of handling on levels in this group (data not shown). In contrast, serum osmolality in the sham-operated animals transferred from ¼ SW to ¼ SW were not significantly ($P>0·05$) different from initial values (data not shown). Serum osmolality in the RPD-autotransplanted animals transferred to ¼ SW from ¼ SW were significantly ($P<0·05$, FPLSD) higher than initial levels, indicating that there may be an effect of handling

on levels in this group. With the exception of the FW-adapted, sham-operated group, serum osmolality increased significantly ($P<0{\cdot}05$) with salinity, with the highest levels occurring in the SW-adapted RPD-autotransplanted (see Fig. 3) and Hx (¼ SW, $327 \pm 1{\cdot}5$; SW, $352 \pm 6{\cdot}8$) groups.

Discussion

The current study presents information on the effects of environmental salinity on circulating prolactin ($tPRL_{177}$ and $tPRL_{188}$) levels and on pituitary $tPRL_{177}$ and $tPRL_{188}$ mRNA(s) levels in hypophysectomized tilapia (*Oreochromis mossambicus*) which have an autotransplanted RPD. Our findings suggest that the expression of the prolactin ($tPRL_{177}$ and $tPRL_{188}$) genes and the secretion of these two hormones are inversely related to plasma osmolality. Our data also show that serum PRL and pituitary PRL mRNA levels in tilapia bearing ectopic pituitary transplants, unlike those of mammals, are nearly equivalent in RPD-autotransplanted and sham-operated animals. The finding that these responses do not require an intact connection between the PRL secreting cells and the hypothalamus is in accord with the elevation of hormone and mRNA levels under conditions that reduce blood osmolality and is appropriate to an endocrine system so closely involved in freshwater osmoregulation (see Bern 1975, Clarke & Bern 1980, Brown & Brown 1987).

We observed higher circulating levels of $tPRL_{177}$ and $tPRL_{188}$ in FW-adapted, sham-operated and RPD-autotransplanted animals, which declined significantly in fish adapted to ¼ SW or SW. In our second study, however, $tPRL_{188}$ levels in the FW-adapted, sham-operated group were not significantly higher (mean levels were higher) than values seen in ¼ SW or SW. We can offer no certain explanation for the low mean levels of $tPRL_{188}$ in the sham-operated group of the second study, although this may reflect differences in handling or sampling procedures of this group. It is interesting, nonetheless, that only $tPRL_{188}$ levels in this group were affected in this manner even though serum osmolality was elevated. Stress may have been the contributing factor, since the animals from study II had shorter post-operative and post-transfer times than those from study I. This is indicated by the increase in serum osmolality (but not circulating PRLs) in the control fish of study II (see Results). One possible mechanism for such an increase in serum osmolality in the FW-adapted sham-operated group of study II is an acute stress-related decrease in plasma volume following handling or confinement (see Okimoto *et al.* 1994, Wendelaar Bonga 1997).

Our examination of pituitary PRL mRNA levels from animals in study I revealed that the patterns of expression in the sham-operated and RPD-autotransplanted groups were similar: levels of $tPRL_{177}$ and $tPRL_{188}$ mRNA expression were highest in the FW-adapted groups and decreased with increasing salinity. Our finding that the gene expression for $tPRL_{177}$ and $tPRL_{188}$ in sham-operated and RPD-autotransplanted tilapia is greater in FW than in SW tilapia (*O. mossambicus*) agrees well with the findings of Nishioka *et al.* (1993). Using *in situ* hybridization techniques, the latter found significantly greater hybridization signals for both $tPRL_{177}$ and $tPRL_{188}$ in the pituitary of FW-adapted tilapia compared with SW-adapted tilapia. Similarly, Auperin *et al.* (1994), using Northern blot procedures, observed decreases in $tPRL_{177}$ and $tPRL_{188}$ gene expression when the stenohaline tilapia, *Oreochromis niloticus*, was transferred from FW to brackish water.

Additional insight into the regulation of PRL can be obtained from our examination of the ratio ($tPRL_{188}$:$tPRL_{177}$) of the two prolactins in both studies. The ratios for serum PRL levels in study I clearly reflect the differential manner in which the two prolactins respond to changes in environmental salinity: the ratio of circulating PRLs was elevated when the fish were adapted to ¼ SW or SW. Our ratios correspond well with the ratios reported in other studies of *O. mossambicus* (Vijayan *et al.* 1996, Morgan *et al.* 1997, Shepherd *et al.* 1997*a*) and *O. niloticus* (Auperin *et al.* 1994). While the ratios of serum prolactins in the groups from study II did not significantly increase in fish adapted to ¼ SW or SW, these values are comparable to ratios seen for *O. mossambicus* in other studies (Ayson *et al.* 1993, Yada *et al.* 1994).

A comparison of the ratios of the two prolactin mRNAs from recovered pituitary tissues of fish from study I showed that the ratio decreases with increasing salinity. This is similar to that which is seen for pituitary content of both PRLs (Borski *et al.* 1992, Ayson *et al.* 1993, Yoshikawa-Ebesu *et al.* 1995) and PRL mRNAs (Nishioka *et al.* 1993) in intact SW-adapted *O. mossambicus*. In contrast, Auperin *et al.* (1994) observed no change in the ratio of prolactin mRNAs in the stenohaline tilapia, *O. niloticus*, transferred from FW to brackish water, although the ratio of the pituitary content of both PRLs did increase. This discrepancy may reflect species or methodological differences. Since the changes in levels of PRL mRNA and their ratios ($tPRL_{188}$:$tPRL_{177}$) from study I agree well with mRNA values reported in other studies of *O. mossambicus*, we believe they have biological relevance even though, for technical reasons, we did not normalize our values to an internal control (e.g. β-actin or 18S rRNA). These results clearly show that the ratios have almost the same value in the sham-operated and RPD-autotransplanted groups, and also agree with previous studies. Additionally, our analyses of the ratios of circulating tilapia PRLs and pituitary PRL mRNAs provide supporting and consistent results for our conclusion that a plasma factor (probably plasma osmolality) directly exerts a regulatory action on PRL release and gene expression in the pituitary in the absence of hypothalamic innervation.

Blood osmolality increased with salinity. Values of blood osmolality such as those observed in our study have been shown to stimulate or inhibit PRL release in tilapia RPD explants *in vitro* (Zambrano *et al.* 1974, Nagahama *et al.* 1975, Grau *et al.* 1981, 1987, Borski *et al.* 1992), and suggest that changes in plasma osmolality directly alter PRL release from the ectopic RPD. It is unlikely that PRL release could have been affected by residual neurohormones (e.g. neuropeptides and monoamines) present in the RPD autotransplants of fish from these studies (see Nishioka *et al.* 1988), owing to the extended period after surgery. Nevertheless, other factors released into the blood may also be involved in the regulation of PRL in the autotransplanted tilapia RPD. This may include neurohormones (e.g. dopamine, thyrotropin-releasing hormone, gonadotropin-releasing hormone, isotocin and vasotocin) released from cells in the vicinity of the transplanted RPD (Nagahama *et al.* 1975, Wigham & Ball 1977, Wigham *et al.* 1977, Urano *et al.* 1994, Weber *et al.* 1997) or osmosensitive changes in circulating levels (e.g. steroids) or brain levels of other hormones as well (e.g. somatostatin, dopamine and serotonin) (Nishioka *et al.* 1988, De Boeck *et al.* 1996).

The participation of the hypothalamus in the regulation of pituitary PRL in the tilapia is established. However, in the work described here, the hypothalamic regulation of pituitary PRL release appears to be minimal, since the patterns of circulating levels of the two PRLs and pituitary levels of the two PRL mRNAs in the sham-operated (intact) and RPD-autotransplanted tilapia are remarkably similar. In support of this, Sukumar *et al.* (1997) recently demonstrated that electrolytic lesions in the hypothalamus had no effect on the pituitary content of PRLs or GH in *O. mossambicus* as measured by polyacrylamide gel electrophoresis. This situation is different from that in the rat, where chronic hyperprolactinemia is observed in animals bearing anterior pituitary grafts, and further supports our contention that small changes in blood or tissue fluid osmolality do not require hypothalamic mediation to regulate PRL release from the pituitary of the euryhaline tilapia, *O. mossambicus*.

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