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?

B S Shepherd¹, T Sakamoto², S Hyodo³, R S Nishioka⁴, C Ball¹, H A Bern⁴ and E G Grau¹

¹Hawaii Institute of Marine Biology and Department of Zoology, University of Hawaii, Kaneohe, Hawaii 96744, USA
²Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739, Japan
³Department of Biology, University of Tokyo at Komaba, Tokyo 153, Japan
⁴Department of Integrative Biology, University of California, Berkeley, California 94720, USA

(Requests for offprints should be addressed to B S Shepherd, Biotechnology Center, University of Connecticut, 184 Auditorium Road, U-149, Storrs, Connecticut 06269–3140, USA)

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.

Journal of Endocrinology (1999) 161, 121–129

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 Oliverreau 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. 1997b), 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. 1997a). The
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, Oliveueau 1969, Ball 1969a,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. 1997a). 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 vivo may also be active in vitro 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 (tPRL177 and tPRL188) 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 tPRL177 and tPRL188 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 mosmol 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 ¾ 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, ¾
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 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.

**Radioimmunoassay and serum osmolarity**

Serum levels of tPRL177 and tPRL188 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 tPRL177 and tPRL188 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 PRL177 and PRL188 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-32P]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 tPRL188 and tPRL177 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 tPRL177.

Relative abundance of PRL mRNA 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. Oligonucleotide probes (45 mers) for tilapia PRL177 and PRL188 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-32P]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 tPRL188 and tPRL177 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 tPRL177.

Relative abundance of PRL mRNA 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),
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 long-term (>3·0 months) ectopic 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 milliosmols (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).

Study II Serum PRL levels and osmolality in sham-operated and RPD-autotransplanted fish of study II. Values for serum osmolality (closed triangles) are means ± S.E.M. and are expressed in milliosmols (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).
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 tPRL177, tPRL188 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 tPRL177, tPRL188 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 tPRL177 or tPRL188 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 tPRL177 and tPRL188 in RPD-autotransplanted fish were significantly (P<0·001, FPLSD) higher in FW than in ¼ SW or SW (Fig. 3). Serum levels of tPRL177 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 tPRL188 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 tPRL188 levels in the FW-adapted, sham-operated group were low (unlike in the RPD-autotransplanted group), the ratio of the two prolactins (tPRL188:tPRL177) 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 tPRL177 or tPRL188 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 tPRL188 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 tPRL177 in the FW-RPD and FW-sham groups were not significantly (P>0·05) different (Fig. 3).

Serum osmolality (milliosmols) 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

Figure 3 Effects of salinity transfer from ¼ SW to FW, ¼ SW or SW on circulating levels of tPRL177 (solid bars) and tPRL188 (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 tPRL188 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 milliosmols (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).
on levels in this group. With the exception of the FW-adapted, sham-operated group, serum osmolality increased significantly \((P<0.05)\) with salinity, with the highest levels occurring in the SW-adapted RPD-autotransplanted (see Fig. 3) and Hx (¼ SW, 327 ± 1.5; SW, 352 ± 6.8) groups.

Discussion

The current study presents information on the effects of environmental salinity on circulating prolactin (tPRL\textsubscript{177} and tPRL\textsubscript{188}) levels and on pituitary tPRL\textsubscript{177} and tPRL\textsubscript{188} mRNA(s) levels in hypophysectomized tilapia \((\textit{Oreochromis mossambicus})\) which have an autotransplanted RPD. Our findings suggest that the expression of the prolactin (tPRL\textsubscript{177} and tPRL\textsubscript{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\textsubscript{177} and tPRL\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{177} and tPRL\textsubscript{188} mRNA expression were highest in the FW-adapted groups and decreased with increasing salinity. Our finding that the gene expression for tPRL\textsubscript{177} and tPRL\textsubscript{188} in sham-operated and RPD-autotransplanted tilapia is greater in FW than in SW tilapia \((\textit{O. mossambicus})\) agrees well with the findings of Nishioka et al. (1993). Using \textit{in situ} hybridization techniques, the latter found significantly greater hybridization signals for both tPRL\textsubscript{177} and tPRL\textsubscript{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\textsubscript{177} and tPRL\textsubscript{188} gene expression when the stenohaline tilapia, \textit{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\textsubscript{188}/tPRL\textsubscript{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 \textit{O. mossambicus} (Vijayan et al. 1996, Morgan et al. 1997, Shepherd et al. 1997a) and \textit{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 \textit{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 \textit{O. mossambicus}. In contrast, Auperin et al. (1994) observed no change in the ratio of prolactin mRNAs in the stenohaline tilapia, \textit{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\textsubscript{188}/tPRL\textsubscript{177}) from study I agree well with mRNA values reported in other studies of \textit{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, isocitron 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.

Acknowledgements

The authors thank A Burch, C Morrey, H Nishioka, B Ron, M Shepherd, S Shimoda, P Wagner and G Weber for their assistance during this study. We would also like to thank Professor T Hirano for critically reading the manuscript. This research was supported in part by a JSPS postdoctoral fellowship to T S, grants from the Hawaii Aquaculture Development Program 35965, National Science Foundation DBC 9104494, Edwin W Pauley Foundation, University of Hawaii Sea Grant College Program (R/AQ-37), SOEST, Institutional Grant # NA36RG0507 (Yr. 27–28) from the National Oceanic and Atmospheric Administration (NOAA), the National Research Initiative Competitive Grants Program/USDA award agreement no. 95–37206-2283 to E G G, University of Hawaii Sea Grant College Program Development Grants nos R/MR–55 PD and E/ET–23PD to B S S through E G G, and grants from Zenyaku Kogyo Co. (Tokyo, Japan), the California State Resources Agency and California Sea Grant College Program (NOAA), NA89AA-D-SG138 R/F–145 to H A B. This manuscript was completed with the support of a postdoctoral fellowship from the National Research Initiative Competitive Grants Program/USDA award agreement no. 97–352206–5094 to B S S. The US government is authorized to reproduce and distribute for governmental purposes. The views expressed here are those of the authors and do not necessarily reflect the views of NOAA, USDA-ARS or any of their sub-agencies. UNIHI-SEAGRANT-JC98–20.

References


Auperin B, Rentier-Delrue F, Martial JA & Prunet P 1994 Evidence that two tilapia (Oreochromis niloticus) prolactins have different osmoregulatory functions during adaptation to a hyperosmotic environment. Journal of Molecular Endocrinology 12 13–24.


Ball JN 1965 A regenerated pituitary remnant in a hypophysectomized killifish (Fundulus heteroclitus): further evidence for the cellular source of the teleostean prolactin-like hormone. General and Comparative Endocrinology 5 181–185.


Ball JN 1969b Prolactin and osmoregulation in teleost fishes: a review. General and Comparative Endocrinology (Suppl 2) 10–25.


Shepherd BS, Ron B, Burch A, Sparks R, Richman NH, Shimoda SK, Stetson MH, Lim C & Grau EG 1997a Effects of salinity, dietary level of protein and 176-nortestosterone on growth hormone (GH) and prolactin (PRL1,2, and PRL1,3) levels in the tilapia, *Oreochromis mossambicus*. *Fish Physiology and Biochemistry* **17**, 279–288.

Shepherd BS, Sakamoto T, Nishioka RS, Richman NH, Mori I, Madsen SS, Chen TT, Hirano T, Bern HA & Grau EG 1997b Somatotropic actions of the homologous growth hormone (Gh) and prolactins in the euryhaline teleost, *Oreochromis mossambicus*. *Proceedings of the National Academy of Sciences of the USA* **94**, 2068–2072.


Urano A, Kubokawa K & Hiraoka S 1994 Expression of the vasotocin and isotocin gene family in fish. *In Fish Physiology: Molecular


Yada T, Hirano T & Grau EG 1994 Changes in plasma levels of the two prolactins and growth hormone during adaptation to different salinities in the euryhaline tilapia, *Oreochromis mossambicus*. *General and Comparative Endocrinology* **93** 214–223.


Received 16 March 1998
Revised manuscript received 9 November 1998
Accepted 3 December 1998