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Evidence that two tilapia (*Oreochromis niloticus*) prolactins have different osmoregulatory functions during adaptation to a hyperosmotic environment

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ABSTRACT

Two forms of prolactin (tiPRL_I and tiPRL_{II}), with only 69% sequence identity, have been previously described in the cichlid fish tilapia (*Oreochromis* species). In the present study we have attempted to investigate the biological activity of these two prolactin forms during adaptation to a hyperosmotic environment. For this purpose, we have developed two highly sensitive (sensitivity: 0.05 ng/ml) and specific (cross-reactivity <0.04%) radioimmunoassays for tiPRL_I and tiPRL_{II}, using recombinant hormones.

When fish were directly transferred from fresh to brackish water, the measured levels of plasma tiPRL_I and tiPRL_{II} dropped abruptly until 12 h after transfer. Thereafter, plasma tiPRL_{II} remained stable (around 0.5 ng/ml) until the end of the experiment, whereas plasma tiPRL_I continued to decrease to undetectable levels. These different patterns of change are reflected in the calculated ratio of plasma tiPRL_{II} to tiPRL_I, which increased from 2–3 in fresh water-adapted fish to over 10 in fish which had spent 3 days or more in brackish water. The pituitary contents of tiPRL_I and tiPRL_{II} varied in a qualitatively similar fashion after transfer to brackish water. The tiPRL_I content

dropped continuously after 12 h, reaching one-twelfth of its initial level after 2 weeks. The pituitary tiPRL_{II} content, on the other hand, did not decrease significantly until day 7, and after a 2-week exposure to brackish water it had only decreased by 50%.

When injected into tilapia adapted to brackish water, both ovine prolactin and recombinant tiPRL_I induced a clear dose-dependent ion-retaining effect. In contrast, the effect induced by tiPRL_{II} treatment was markedly smaller and not dose-dependent.

Northern blot analysis of tiPRL mRNAs using either a tiPRL_I or a tiPRL_{II} cDNA probe indicated the presence of two mRNAs differing in size: a 1.7 kb mRNA coding for tiPRL_I and a 1.3 kb mRNA coding for tiPRL_{II}. After transfer to brackish water, levels of the two mRNAs decreased similarly.

The present study indicates that, in *O. niloticus*, the two forms of prolactin have different osmoregulatory roles during adaptation to brackish water. Accordingly, their syntheses are differentially regulated after transfer to a hyperosmotic environment, presumably at a post-transcriptional level.

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INTRODUCTION

Prolactin belongs to a family of structurally and functionally related polypeptides which also includes growth hormone (GH), placental lactogen, proliferin and somatolactin. Although in man, rats and turkeys prolactin is the product of a single gene, the hormone occurs in multiple molecular forms (cleaved, phosphorylated or glycosylated; Lewis *et al.* 1984; Clapp *et al.* 1989; Brooks *et al.* 1990), whose discrete functions remain a matter of debate.

In teleost fish, nucleotide and polypeptide sequence analyses have revealed the existence of two distinct, albeit similar, genes in some species. Such is the case for salmon, *Oncorhynchus keta*, and for carp, *Cyprinus carpio* (Yasuda *et al.* 1986, 1987). In tilapia, two much less similar prolactin molecules have been characterized; their sequence identity is only 69%, one variant (tilapia prolactin I, tiPRL_I) being more similar to other fish prolactins than to the other variant (tiPRL_{II}) (Yamaguchi *et al.* 1988; Rentier-Delrue *et al.* 1989).

In fish, prolactin exhibits a pleiotropic spectrum of biological activities including effects on osmoregulation, metabolism and behaviour (for a review see Clarke & Bern, 1980). However, the primary and most studied role of prolactin in fish is water and electrolyte homeostasis (Loretz & Bern, 1982; Hirano, 1986; Prunet *et al.* 1990). In fresh water, treatment of hypophysectomized tilapia (*Oreochromis mossambicus*) with mammalian or fish prolactin restores the osmolality and electrolyte concentrations to normal levels (Dharmamba *et al.* 1967; Dharmamba, 1970; Specker *et al.* 1985), thus demonstrating that prolactin is necessary for survival in a hypo-osmotic environment. Furthermore, when injected into sea water-adapted tilapia, ovine prolactin profoundly disturbs the hydromineral balance by inhibiting salt extrusion by gill chloride cells (Dharmamba & Maetz, 1972; Dharmamba *et al.* 1975; Foskett *et al.* 1983).

Both tiPRLs behave similarly in restoring the hydromineral balance of hypophysectomized tilapia transferred to fresh water (Specker *et al.* 1985; Young *et al.* 1988). However, Specker *et al.* (1989) have shown that the salamandrid integumental bioassay can distinguish between the two prolactin variants.

To analyse possible differences in biological function between the two variants further, we have produced recombinant tiPRLs and used multiple approaches to study their osmoregulatory roles during adaptation to a hyperosmotic environment. With radioimmunoassays (RIAs) specific for each form of prolactin, we have measured plasma and pituitary levels after direct transfer from fresh water to brackish water. We additionally analysed both tiPRL_I and tiPRL_{II} mRNAs. We also report the effects of injected tiPRL_I and tiPRL_{II} on electrolyte homeostasis in tilapia adapted to brackish water. Our results clearly demonstrate that the two prolactins perform different biological functions during adaptation to a hyperosmotic environment, and that they are differentially regulated.

MATERIALS AND METHODS

Sources of hormones and antisera

Recombinant tiPRL_I (large tiPRL, 188 amino acids), tiPRL_{II} (small tiPRL, 177 amino acids) and tilapia GH were produced in *Escherichia coli* and further purified (Rentier-Delrue *et al.* 1989; Swennen *et al.* 1991). Ovine prolactin (NIDDK-oPRL-19) was supplied by the National Institute of Arthritis and Metabolic Diseases, NIH, Bethesda, MD, U.S.A. Chinook salmon prolactin was purified according to Prunet & Houdebine (1984). Antisera

against recombinant tiPRL_I and tiPRL_{II} were raised in rabbits.

Iodination of tiPRL

The two tiPRLs were iodinated according to the chloramine-T method (Greenwood *et al.* 1963) modified by Martal (1972). Specific activities were estimated (about 40–50 µCi/µg for tiPRL_I and 50–60 µCi/µg for tiPRL_{II}) from the rate of iodine incorporation into prolactin. Labelled hormone was stored in 50% glycerol at –20 °C.

Radioimmunoassay procedure

The same procedure was used for both tiPRL_I and tiPRL_{II}. RIAs were performed using a double antibody method under non-equilibrium conditions as described by Prunet *et al.* (1985), with minor differences. The second precipitation was obtained by adding sheep anti-rabbit gamma globulin antiserum to each tube without polyethylene glycol.

In order to validate these RIAs, plasma and pituitaries from different tilapia species (*O. niloticus*, *O. aureus* and *Sarotherodon melanotheron*) were collected on the Ivory Coast from adult fish. Moreover, plasma from hypophysectomized adult (250–300 g) *O. niloticus* specimens was also tested. Hypophysectomy was performed as described by Nishioka (1980) and blood was collected 24 and 48 h after surgery.

Transfer experiment

Tilapia specimens (*O. niloticus*) supplied by IDESSA (Bouaké, Ivory Coast) were reared in recirculating fresh water at a constant temperature (26 °C) and with a constant photoperiod (12 h light:12 h darkness) for 7 months.

Two similar experiments were performed, one over a 14-day period (with fish having a weight of 91 ± 1 g (mean $\pm$ S.E.M., $n=8$)) and another over a 28-day period (long-term adaptation, with fish having a weight of 134 ± 3 g). Fish were transferred to aquaria (eight fish per 80 litre aquarium with filter) and kept in fresh water for 2 weeks before starting the experiment. Transfer to brackish water (20‰ salinity) was effected by adding an adequate amount of concentrated salt water made with marine salt. Thus, transfer to the hyperosmotic environment was nearly instantaneous. The water temperature during the experiment was the same in the fresh water and brackish water aquaria (26–27 °C) and the photoperiod was 12 h light:12 h darkness in both cases. Neither group was starved after transfer. Two-thirds of the water was changed twice a week.

Eight fish from a fresh water aquarium and eight from a brackish water aquarium were sampled at each point in the experiment. On the day of transfer, a sample of seven fish, representative of the whole fresh water population, was taken as a control group.

Plasma and pituitary collection

To avoid the effect of stress on plasma prolactin levels, the fish were caught individually by hand-net in each aquarium. This technique proved to be preferable to anaesthesia, as the latter significantly increases the plasma levels of tiPRL_I and tiPRL_{II} (B. Auperin & P. Prunet, unpublished results). Within 1 min of catching each fish, and before collecting the pituitary, blood was collected by caudal puncture with a heparinized syringe. After blood centrifugation, plasma was collected and frozen at -20°C . Pituitaries were immediately frozen in liquid nitrogen and stored at -20°C until assayed.

Hormonal treatment of *O. niloticus* adapted to brackish water

Tilapia (*O. niloticus*), with a mean weight of 20 ± 0.3 g and adapted to an aquarium, were directly transferred to brackish water (20‰ salinity) at a constant temperature of 27°C , and maintained at this salinity for 2 weeks prior to the start of the experiment. No mortality was observed during the period of adaptation and fish were fed at a rate of 1% body weight per day before and during the experiment. Fish were further distributed into four aquaria (capacity 80 litres; 9 or 10 fish per aquarium) and injected intraperitoneally every 2 days for 8 days. On the day after the last injection, blood was collected, centrifuged and frozen at -20°C until analysis.

Each group of fish received one of the following injections: saline (NaCl, 0.15 M in 1 ml for 50 g fish); recombinant tiPRL_I (0.1, 0.25 or 0.5 µg/g body weight, dissolved in 10 mM NH_4HCO_3 (1 µg/2 µl) and diluted with 0.15 M NaCl to the desired concentration); recombinant tiPRL_{II} (same concentrations as for tiPRL_I); ovine prolactin (4, 10 or 20 µg/g body weight, prepared in the same manner as tiPRL). In order to test the different doses mentioned above, three independent experiments were performed at the same time with fish from the same stock.

Ion measurements

The chloride level was measured by amperometric titration (Isa Biologie CL 500; Isa Biologie,

Morangis, France) of 20 µl undiluted plasma. The plasma sodium level was determined by absorption spectrophotometry using plasma samples diluted 4000 times.

RNA analysis

Pituitaries from tilapia (*O. niloticus*) reared in fresh water or adapted over 2 weeks to brackish water (20‰ salinity) were collected and immediately frozen in liquid nitrogen.

Total RNA was prepared according to the method of Chomczynski & Sacchi (1987). Tilapia mRNA was further size-fractionated on formaldehyde-agarose gels, and then transferred to nylon membranes (Biodyn-Pall; Pall Europe Ltd, Portsmouth, Hants, U.K.). All hybridization was performed under conditions of high stringency (Thomas, 1980). The probes used in these hybridization experiments were the full-length (coding and non-coding sequences) tiPRL_I and tiPRL_{II} cDNAs inserted into pUC13 and cut at the EcoRI sites as described by Rentier-Delrue *et al.* (1989). We kept both coding and non-coding sequences in order to have most dissimilarity between the two tiPRLs. To control for variation in sample loading, the nylon membranes were further stripped and reprobed for β -actin mRNA using rainbow trout cDNA (Pakdel *et al.* 1989). All cDNAs were labelled with a random-priming DNA labelling kit and [α - ^{32}P]dCTP (800 Ci/mmol; Amersham, Les Ulis, France). The band intensities were quantified by densitometric scanning of autoradiograms. The two mRNA species (1.3 and 1.7 kb) revealed on blots hybridized with either the tiPRL_I cDNA or the tiPRL_{II} cDNA probe were scanned, and quantification of these bands allowed us to calculate the ratio of tiPRL_{II} mRNA to tiPRL_I mRNA for different pituitary samples. This quantification was performed with samples showing different quantities of tiPRL mRNAs and with different amounts of total RNA (3 or 5 µg) loaded on the gel. Autoradiograms were exposed for several periods and scanning analysis was performed on films where signals were not saturated.

Statistical analysis

The Mann-Whitney test was used to characterize differences between the means from experiments involving treatment or transfer, after variance analysis with nested factors (analysis of variance, with transfer or treatments and time as factors). Results are presented as means $\pm$ S.E.M. Significance was accepted at the 5% level.

FIGURE 1. Radioimmunoassays for recombinant (a) tilapia prolactin I (tiPRL_I) and (b) tiPRL_{II}, showing the displacement of ^{125}I -labelled tiPRL_I or ^{125}I -labelled tiPRL_{II} by the tiPRL_I (■) and tiPRL_{II} (▲) standards, different hormonal preparations (tilapia GH, ovine PRL, salmon PRL; ○), pituitary homogenate (dotted lines) and plasma (dashed lines) sample dilutions (as shown) from different fresh water-reared Cichlid species (*Oreochromis niloticus* (○), *O. aureus* (▽) and *Sarotherodon melanotheron* (□)) and plasma from hypophysectomized *O. niloticus* (▽). Each point is the mean of duplicate determinations. ^{125}I -Labelled PRL bound (%) refers to ^{125}I -labelled PRL bound as a percentage of the label bound in the absence of tiPRL.

RESULTS

Validation of the RIAs for tiPRL_I and tiPRL_{II} (Fig. 1)

When tested in the corresponding RIAs, serial dilutions of pituitary extracts and plasma from *O. niloticus*, *O. aureus* and *S. melanotheron* produced

inhibition curves that paralleled the standard curves for tiPRL_I or tiPRL_{II}. Plasma from hypophysectomized *O. niloticus* failed to displace labelled hormone in both RIAs. Assay specificity was further confirmed by the absence of cross-reactivity with tilapia GH, ovine prolactin and salmon prolactin. Furthermore, tiPRL_{II} did not cross-react in the tiPRL_I RIA, nor did tiPRL_I exhibit significant cross-reactivity in the tiPRL_{II} RIA (0.04% for a 50% displacement).

The mid-range value, calculated as the amount of tiPRL required to bind 50% of the zero-hormone standard (ED₅₀) was 0.493 ± 0.013 ng/ml for tiPRL_I and 0.938 ± 0.029 ng/ml for tiPRL_{II}. The estimated assay sensitivity, defined as twice the S.D. at zero dose, was 0.05 ng/ml in both assays. These values tally well with the plasma levels of both prolactins.

The effects of plasma components on the assay were examined by determining the recovery of tiPRL_I or tiPRL_{II} in the presence of 50 µl plasma from hypophysectomized *O. niloticus*. The slope of the regression line between prolactin added and recovered did not differ significantly from 1 when the amounts used did not exceed 4 ng/ml in the tiPRL_I RIA or 5 ng/ml in the tiPRL_{II} assay. Above these concentrations, the prolactin concentration was slightly underestimated (data not shown). Repeated determinations of a pool of *O. niloticus* plasma gave intra- and interassay coefficients of variation of 8.0% ($n=10$) and 10.5% ($n=4$) for tiPRL_I and 7.3% ($n=10$) and 12.2% ($n=4$) for tiPRL_{II} respectively.

Changes in plasma and pituitary prolactin levels during adaptation to brackish water (Figs 2 and 3)

Within 3 h of direct transfer to brackish water, plasma Cl⁻ levels were found to have increased significantly (data not shown). The highest levels were observed on the day after transfer (day 1) (200 ± 3.7 versus 138 ± 1.5 mEq/l in fresh water

FIGURE 2. Changes in plasma (a) tilapia prolactin I (tiPRL_I) and (b) tiPRL_{II} levels and (c) the plasma tiPRL_{II}:tiPRL_I ratio of *Oreochromis niloticus* after direct transfer from fresh water to 20‰ brackish water (triangles) or retention in fresh water (squares). Values shown are means $\pm$ S.E.M. ($n=8$ or 9). * $P < 0.05$, ** $P < 0.01$ compared with values for fresh water fish (the Mann-Whitney test was used to characterize differences between means after variance analysis with nested factors, with transfer and time as factors). ND: no detectable values.

fish, $n=8$). From then on, however, the Cl^- levels dropped, stabilizing at 10% above the fresh water values on day 3. This shows that the fish were able to regulate their hydromineral balance. Plasma Na^+ levels exhibited the same pattern of change (data not shown).

As early as 6 h after exposure to brackish water, the levels of both plasma prolactins dropped abruptly. This trend continued until 12 h after transfer, after which the tiPRL_{II} level remained low (0.5 ng/ml) until the end of the experiment (on day 14), whereas the tiPRL_I level continued to drop. By day 7, tiPRL_I was undetectable and remained so on day 14. In the long-term adaptation experiment, the hormone levels on day 28 (0.13 ± 0.01 ng/ml for tiPRL_I and 1.058 ± 0.155 ng/ml for tiPRL_{II}) did not differ significantly from those measured on day 14 (0.12 ± 0.002 and 0.66 ± 0.107 ng/ml respectively).

The calculated ratio of plasma tiPRL_{II} to plasma tiPRL_I clearly reflects these differences in the way the two levels change after exposure to brackish water. From a value of between 2 and 3 in fresh water, this ratio rose to over 10 after a 3-day exposure.

In the transfer experiment, fish weights remained fairly homogenous (less than 8% variability), so prolactin contents per pituitary could be directly compared in the different groups. Twelve hours after transfer, a significant drop in pituitary tiPRL_I was observed. Thereafter, the tiPRL_I content continued to decrease, and after a 2-week exposure to brackish water it was 12 times lower than the values observed in fresh water controls. Changes in tiPRL_{II} content after transfer followed a different pattern; the tiPRL_{II} content did not drop significantly until 7 days after transfer. By the end of the experiment, the tiPRL_{II} content had decreased by only half when compared with the control group. In long-term-adapted fish (28 days) the prolactin contents (0.465 ± 0.02 μg/pituitary for tiPRL_I and 3.762 ± 0.627 μg/pituitary for tiPRL_{II}) did not differ significantly from those measured in the same experiment on day 14 (0.586 ± 0.102 and 4.057 ± 1.040 μg/pituitary respectively).

Here again, the tiPRL_{II}:tiPRL_I ratio reflects how differently the amounts of tiPRL_I and tiPRL_{II} in the pituitaries changed in response to transfer. This ratio increased from 1 before transfer to 5 after adaptation to brackish water.

FIGURE 3. Changes in pituitary (a) tilapia prolactin I (tiPRL_I) and (b) tiPRL_{II} levels and (c) the pituitary tiPRL_{II}:tiPRL_I ratio of *Oreochromis niloticus* after direct transfer from fresh water to 20‰ brackish water (triangles) or retention in fresh water (squares). Values shown are means $\pm$ S.E.M. ($n=8$ or 9). * $P < 0.05$, ** $P < 0.01$ compared with values for fresh water fish (the Mann-Whitney test was used to characterize differences between means after variance analysis with nested factors, with transfer and time as factors).

FIGURE 4. Northern blot analysis of mRNA extracted from 20 fresh water-adapted tilapia pituitaries. Total RNA (5 µg) was denatured, electrophoresed and transferred to nylon membranes. The filter was successively hybridized with tilapia prolactin I (tiPRL_I) cDNA (panel 1) and tiPRL_{II} cDNA (panel 2) (exposure times: tiPRL_I, 2 days; tiPRL_{II}, 4 days). A control autoradiograph was made after each dehybridization. The sizes of RNA molecular markers (kb) are indicated on the left.

FIGURE 5. Northern blot analysis of mRNA extracted from 20 pituitaries collected from fresh water-adapted (F) and brackish water-adapted (B) tilapia (*Oreochromis niloticus*). Total RNA (5 µg) was denatured, electrophoresed and transferred to nylon membranes. The filter was successively hybridized for tilapia prolactin II (tiPRL_{II}) (panel 1), tiPRL_I (panel 2) and actin (panel 3) (exposure times: tiPRL_{II}, 6 days; tiPRL_I, 7 days; actin, 8 days). A control autoradiograph was made after each dehybridization.

Analysis of tiPRL mRNA (Figs 4, 5 and 6)

Tilapia prolactin mRNAs were analysed by Northern blotting, and the same membranes were successively hybridized with tiPRL_{II} and tiPRL_I

FIGURE 6. Ratio of tilapia prolactin II (tiPRL_{II}) mRNA to tiPRL_I mRNA in tilapia adapted to fresh water or transferred for 2 weeks to brackish water. Total RNA was extracted from ten pituitaries collected from fish adapted to fresh or brackish water. Pituitary PRL mRNAs were measured after Northern blot analysis using tiPRL_I cDNA as a probe and autoradiography. Values are given as means ± S.E. of three independent Northern blots corresponding to three independent transfer experiments. The ratio in brackish water was not significantly different from that in fresh water (Mann-Whitney non-parametric test).

cDNA probes. Using conditions of high stringency, two bands of different sizes (1.7 and 1.3 kb) and intensities were observed with each probe (Fig. 4). Interestingly, whereas for tiPRL_I cDNA the more intense signal corresponded to the 1.7 kb mRNA, the tiPRL_{II} probe gave the strongest signal with the 1.3 kb mRNA. As hybridization was performed under conditions of high stringency, we conclude that the 1.7 kb mRNA was tiPRL_I mRNA, whereas the 1.3 kb species was the tiPRL_{II} mRNA. However, these results also indicate that the tiPRL_I and tiPRL_{II} cDNAs hybridized with both mRNA species. When comparing fish adapted to fresh water and those adapted to brackish water, a large decrease in both tiPRL_I and tiPRL_{II} mRNA was observed (Fig. 5). We were further interested to compare the ratio of tiPRL_{II} mRNA to tiPRL_I mRNA in tilapia adapted to fresh water and brackish water. Calculated from Northern blots hybridized with tiPRL_I cDNA, this ratio did not change significantly after exposure to brackish water, thus indicating that both mRNAs decreased

FIGURE 7. Effect of different doses of recombinant tilapia prolactin I (tiPRL_I), recombinant tiPRL_{II} and ovine (o)PRL on plasma (a) chloride and (b) sodium values. Values are shown as means $\pm$ S.E.M. ($n=8$ or 9). ** $P<0.01$ compared with control; †† $P<0.01$, tiPRL_{II} treatment vs corresponding treatment with tiPRL_I (Mann-Whitney non-parametric test). For each treatment, bar 1 represents the low dose (oPRL, 4 μ g/g; tiPRL_I or tiPRL_{II}, 0.1 μ g/g), bar 2 represents the medium dose (oPRL, 10 μ g/g; tiPRL_I or tiPRL_{II}, 0.25 μ g/g) and bar 3 represents the high dose (oPRL, 20 μ g/g; tiPRL_I or tiPRL_{II}, 0.5 μ g/g). mEq: milliEquivalent.

similarly after transfer (Fig. 6). A similar result was observed when using blots hybridized with tiPRL_{II} cDNA (data not shown).

Effect of prolactin treatment in *O. niloticus* adapted to brackish water (Fig. 7)

When injected into tilapia specimens adapted to brackish water, both ovine prolactin and recombinant tiPRL_I induced a clearly dose-

dependent ion-retaining effect, as indicated by the increased plasma Na⁺ and Cl⁻ levels. Furthermore, tiPRL_I was between 50 and 100 times more potent than ovine prolactin. In contrast, tiPRL_{II} only slightly increased the plasma Na⁺ and Cl⁻ levels. The effect was not dose-dependent and was significantly smaller than the effect of either ovine prolactin or tiPRL_I.

DISCUSSION

Since the first description of two prolactins displaying only 69% similarity in their amino acid sequences in the fish species *Oreochromis* (Specker *et al.* 1985; Yamaguchi *et al.* 1988; Rentier-Delrue *et al.* 1989), several unsuccessful attempts have been made to distinguish the two prolactin molecules on a functional basis.

The availability of large amounts of active recombinant tiPRLs showing full biological activity (Swennen *et al.* 1991) has enabled us to investigate differences in the control of osmoregulation by the two prolactins during adaptation to a hyperosmotic environment. To this end, we have developed a highly specific RIA for each prolactin. The two prolactins being quite similar (69%), it was necessary to control the specificity of each RIA carefully. No significant cross-reactivity (<0.04%) was observed between the antisera raised against tiPRL_I and tiPRL_{II}. Furthermore, no cross-reaction was observed with either tilapia GH or plasma from hypophysectomized fish, whereas the displacement curves of plasma and pituitary extracts from various cichlid species were parallel to the standard curves in both assays. Importantly, both RIAs are very sensitive, as shown by their ED₅₀ values and the lowest detectable concentrations. They appear to be at least five times more sensitive than previously reported RIAs for fish prolactins or GHs (Nicoll *et al.* 1981; Hirano *et al.* 1985; Prunet *et al.* 1985; Le Bail *et al.* 1991; Suzuki & Hirano, 1991; Ayson *et al.* 1993). This confirms that our RIAs, developed with antisera raised against recombinant tiPRL_I and tiPRL_{II}, are perfectly suitable for measuring plasma and pituitary levels of both tiPRLs.

These RIAs enabled us to investigate the osmoregulatory functions of both prolactins during the adaptation of *O. niloticus* to a hyperosmotic environment. Our first approach was to determine plasma and pituitary levels of both forms at various times prior to and after transfer from fresh to brackish water. As described for several fish species (Nicoll *et al.* 1981; Hirano *et al.* 1985; Prunet *et al.* 1985; Suzuki & Hirano, 1991; Ayson *et al.* 1993),

plasma prolactin levels are higher in fish kept in fresh water than in ones adapted to a hyperosmotic environment. Our results, however, highlight interesting differences between the two forms. Although both prolactin levels rapidly decreased upon transfer to brackish water, their patterns of change differed; the plasma tiPRL_I level continued to drop after the first day, reaching undetectable or barely detectable levels by the seventh day, while the plasma tiPRL_{II} level, after an initial drop during the first half-day, then stabilized near 0.5 ng/ml and remained practically constant, even after 28 days in brackish water. The following features emerge from our results. Under the conditions used (1) the plasma level of tiPRL_{II} was always higher than that of tiPRL_I, (2) the plasma tiPRL_{II}:tiPRL_I ratio increased markedly after transfer, (3) in fish adapted to brackish water, tiPRL_I was no longer detectable in the plasma whereas a significant level of tiPRL_{II} could still be measured. We stress that such differences can only be observed with very sensitive and specific assays, such as the two RIAs developed in the present study. Ayson *et al.* (1993) reported a low but significant plasma level of tiPRL_I in sea water-adapted tilapia (*O. niloticus*) and, in their experiments, they observed a decreased ratio of plasma tiPRL_{II} to tiPRL_I after transfer to brackish water. Beyond possible problems of assay sensitivity, these data might have been hampered by sampling stress; 2-phenoxyethanol, used by Ayson *et al.* (1993) as an anaesthetic, has been shown to strongly stimulate plasma prolactin levels in tilapia (B. Auperin & P. Prunet, unpublished observations). Thus, these data cannot really be compared with our results obtained under non-stressful conditions.

The amounts of tiPRL_I and tiPRL_{II} in the pituitaries also exhibited different patterns of change after transfer. A drop in tiPRL_I content was observed between the first half-day and the third day after transfer, with a sharper drop by day 7 and thereafter. By day 14, the tiPRL_I content was only one-twelfth of its initial value. The tiPRL_{II} content, on the other hand, had decreased only by half after 2 weeks in brackish water. The fact that tiPRL_{II}, unlike tiPRL_I, is present at significant levels in fish adapted to brackish water suggests that the two prolactins have different osmoregulatory roles during adaptation to a hyperosmotic environment.

To test this hypothesis further, we investigated the effects of repeated injections of recombinant tiPRL_I or tiPRL_{II} in fish adapted to brackish water. Interestingly, tiPRL_I behaved like ovine prolactin in that it induced a dose-dependent ion-retaining effect, as shown by the increased Na⁺ and Cl⁻ levels. This is in keeping with results obtained with

ovine prolactin or tilapia pituitary extracts (Clarke, 1973a,b; Dharmamba & Maetz, 1976). In the present study, tiPRL_I was between 50 and 100 times more potent than ovine prolactin. The increase caused by tiPRL_{II}, on the other hand, was markedly smaller and not dose-related. Since the two tiPRL forms produced in *E. coli* exhibit similar potencies in two different bioassays and are as biologically active as natural tiPRLs (Swennen *et al.* 1991; Auperin *et al.* 1993), the results obtained in this particular test cannot be attributed to differences in the ability of the two recombinant prolactins to perform their biological functions. Moreover, as suggested by Swennen *et al.* (1991), Auperin *et al.* (1993) have recently demonstrated the presence of only one class of tiPRL receptor in gill and kidney, which binds tiPRL_I with a higher affinity than tiPRL_{II}. This allowed us to exclude the hypothesis that the low endogenous plasma levels of tiPRL_{II} would have interfered (at the level of receptor) with exogenous tiPRL treatment; in such a case, a similar effect would have been observed with both tiPRL forms. We conclude that the significant level of tiPRL_{II} measured in fish adapted to a hyperosmotic environment can be linked with the fact that this hormone exerts a limited ion-retaining effect. This further tallies with the observation that tiPRL_I, which affects the hydro-mineral balance much more drastically, is undetectable under the same conditions. We conclude from this that the two prolactins have different osmoregulatory roles in *O. niloticus* adapted to brackish water.

Previous studies comparing the osmoregulatory effects of the two tiPRLs used biological assays based on the physiology of fresh water osmoregulation, including the Na⁺-retaining effect in hypophysectomized tilapia (*O. mossambicus*) reared in fresh water (Specker *et al.* 1985; Young *et al.* 1988), effects on transepithelial potential measurements in hypophysectomized tilapia (Young *et al.* 1988), and effects on plasma Ca²⁺ levels (Swennen *et al.* 1991). In all cases, both forms exhibited similar biological activities and their osmoregulatory effects resembled those described for ovine prolactin (Dharmamba, 1970; Wendelaar Bonga & Meis, 1981; Flik *et al.* 1984). A comparison with our results suggests that prolactin may exert different effects in hyperosmotic and hypo-osmotic environments, and that some of these effects may be mediated by one form only.

In tilapia adapted to sea water, ovine prolactin inhibits chloride secretion by the gills (Foskett *et al.* 1982) and reduces branchial efflux (Dharmamba & Maetz, 1976), which results in an increased Na⁺ level. Accordingly, Pisam *et al.*

(1993) have recently shown that tiPRL_I significantly alters the ultrastructural features of gill chloride cells shifted from a 'sea water' to a 'fresh water' type. Together with the present study, these data indicate that tiPRL_I modifies gill chloride cell differentiation and the activity of these cells in a hyperosmotic environment. A similar effect was also observed after ovine prolactin injection (M. Pisam, unpublished observation). On the other hand, our present data on tiPRL_{II} and the limited effect of this form on chloride cell morphology (M. Pisam, unpublished observation) suggest that tiPRL_{II} might not be involved in the regulation of chloride cells. Foskett *et al.* (1983) have suggested that prolactin may not act on chloride cells in fresh water-adapted tilapia; the Na⁺-retaining effect of prolactin traditionally described in fresh water-reared specimens could be due to a decreased osmotic permeability to water in osmoregulatory organs (Dharmamba & Maetz, 1972; Ogawa *et al.* 1973; Wendelaar Bonga & Van Der Meij, 1980). According to Specker *et al.* (1985), both forms of tiPRL prevent the loss of Na⁺ from the plasma of hypophysectomized tilapia kept in fresh water. In conclusion, we suggest that tiPRL_{II}, in contrast to tiPRL_I, does not affect chloride cells in a hyperosmotic environment, but does inhibit permeability to water, which could explain the slight but significant ion-retaining effect observed.

Upon transfer to brackish water, the ratio of tiPRL_{II} to tiPRL_I was found to increase markedly in the pituitaries and plasma of transferred fish. This points to a differential regulation of the two forms in response to salinity changes, as regards their synthesis in the pituitary and subsequent release. This is in keeping with electrophoretic studies of pituitary tiPRL_I and tiPRL_{II} contents in fresh water- and sea water-adapted *O. mossambicus* (Borski *et al.* 1992). However, no differences were detected in the neuroendocrine control of synthesis or release of the two tiPRLs. Likewise, both tiPRL forms responded similarly when the actions of osmotic pressure and neuropeptides were tested on pituitary fragments (Rivas *et al.* 1986; Kelley *et al.* 1988). Clearly, it is necessary to conduct further studies on tilapia adapted to a hyperosmotic environment in order to clarify which endocrine factors mediate this differential regulation.

Further insight into the differential regulation of tiPRL synthesis was provided by our analysis of prolactin mRNA in *O. niloticus* adapted to fresh water and brackish water. Although both tiPRL cDNA probes hybridized with both mRNAs, differences in size and intensity enabled us to conclude that tiPRL_I and tiPRL_{II} are the respective translation products of the large (1.7 kb) and the

small (1.3 kb) mRNAs. Environmental salinity modified the pituitary content of both tiPRL mRNAs as indicated by Northern blot analysis. This is in keeping with semi-quantitative *in situ* hybridization analysis of tiPRL mRNA in *O. mossambicus* transferred to sea water (Nishioka *et al.* 1993). Interestingly, after a 2-week exposure to brackish water in the present study, both mRNA levels were found to have decreased similarly, despite the fact that the tiPRL_{II} content was five times higher than the tiPRL_I content in the pituitaries of these fish (Fig. 3). This suggests that the differential regulation of the two forms occurs primarily at one or more post-transcriptional levels. Post-transcriptional regulation of prolactin synthesis has been described in the literature. In GH₃ cells, treatment with Ca²⁺ significantly increases the level of prolactin mRNA by regulating post-transcriptional steps occurring very soon after the completion of transcription (Preston *et al.* 1990; Delidow *et al.* 1992). This does not affect the half-life of prolactin mRNA in the cytoplasm. In contrast, Gellersen *et al.* (1992) have observed an increase in steady-state levels of human prolactin in the IM-9-P3 cell line due to mRNA stabilization after retinoic acid treatment.

In conclusion, our evidence suggests that the two tiPRLs perform different osmoregulatory functions in *O. niloticus* adapted to brackish water. It further points to differential regulation of tiPRL_I and tiPRL_{II} synthesis, probably at a post-transcriptional level, after transfer to a hyperosmotic environment.

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