

Expression of prolactin-releasing peptide and prolactin in the euryhaline mudskippers (*Periophthalmus modestus*): prolactin-releasing peptide as a primary regulator of prolactin

T Sakamoto^{1,2}, M Amano³, S Hyodo⁴, S Moriyama³, A Takahashi³, H Kawauchi³ and M Ando¹

¹Ushimado Marine Laboratory, Faculty of Science, Okayama University, Ushimado, Setouchi 701-4303, Japan

²Faculty of Integrated Arts and Sciences, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739-8521, Japan

³School of Fisheries Sciences, Kitasato University, Sanriku, Ofunato 022-0101, Japan

⁴Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164-8639, Japan

(Requests for offprints should be addressed to T Sakamoto, Ushimado Marine Laboratory, Faculty of Science, Okayama University 130-17, Kashino, Ushimado, Setouchi, 701-4303, Japan; Email: ryu@uml.okayama-u.ac.jp)

Abstract

Prolactin (PRL)-releasing peptide (PrRP) is a strong candidate stimulator of pituitary PRL transcription and secretion in teleosts. However, the role in control of extrapituitary PRL expression is unclear even in mammals. To study the possible presence of PrRP–PRL axes not only in the brain-pituitary but also in peripheral organs, the expression patterns of PrRP, PRL and growth hormone (GH) were characterized in amphibious euryhaline mudskippers (*Periophthalmus modestus*). PrRP mRNA is abundantly expressed not only in the brain but also in the liver, gut and ovary, while less abundant expression was also detected in the skin and kidney. Corresponding to the distribution of PrRP mRNA, PRL mRNA was also detectable in these organs. During adaptation to different environments, the changes in mRNA levels of PrRP paralleled those in PRL in the brain-pituitary, liver and gut in an organ-specific manner. Brain PrRP mRNA and the pituitary PRL mRNA increased under freshwater and terrestrial conditions ($P < 0.05$); expression of PrRP and PRL in the gut of freshwater fish was higher ($P < 0.05$) than those in sea-water fish although there were no changes in fish kept out of water; no significant change was seen in the liver. Expressions of GH were not correlated with PrRP. In the gut, PrRP and PRL appear to be co-localized in the mucosal layer, especially in the mucous cells. Thus, PrRP may also be a local modulator of extrapituitary PRL expression and the PrRP–PRL axes in various organs may play an organ-specific role during environmental adaptation.

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Introduction

Although prolactin (PRL) is historically known as a pituitary hormone; however, in the past few years, interest has been raised in locally produced, extrapituitary PRL. The current view of the regulation of pituitary PRL expression integrates a wide spectrum of molecules. However, the control of extrapituitary PRL expression is still poorly understood, both at the transcriptional and at the secretory levels (Goffin *et al.* 2002). Since extrapituitary PRL expression in trout, goldfish and seabream has been reported to be relatively high, unlike that in tetrapods (Santos *et al.* 1999, Yang *et al.* 1999, Imaoka *et al.* 2000), studies using aquatic teleosts should shed new light on the regulation of extrapituitary PRL in terrestrial vertebrates.

PRL-releasing peptide (PrRP) is a peptide identified from mammalian hypothalamus with a specific PRL-

releasing activity on pituitary cells (Hinuma *et al.* 1998, Sakamoto *et al.* 2003a). Concurrently, Fujimoto *et al.* (1998) isolated a homologue of PrRP from the Japanese crucian carp, *Carassius auratus langsdorfi*. Recently, homologues have also been isolated from tilapia and chum salmon, showing these peptides to be identical to the carp peptide (Moriyama *et al.* 2002, Seale *et al.* 2002). Although there are contradictory data on PRL-releasing effects in rats, PrRP appears to be a potent hypothalamic secretagogue for PRL as well as an inducer of PRL transcription in teleost pituitaries (Sakamoto *et al.* 2003a). On the other hand, both in fish and in mammals, PrRP appears to inhibit secretion of growth hormone (GH; Sakamoto *et al.* 2003a), another member of the hormone family sharing a common ancestral gene with PRL (Rand-Weaver *et al.* 1993). In mammals, PrRP is expressed in the central nervous system, but is also produced in the digestive tract, kidney, pancreas,

adrenal gland, gonads and decidua (Fujii *et al.* 1999). Although PrRP is suggested to be a local stimulator of decidal PRL release in humans (Reis *et al.* 2002), the tissue distribution of PrRP in non-mammalian species is unknown.

In teleosts, the most prominent action of PRL is osmoregulation in fresh water. Among a number of activities associated with PRL through vertebrates, such osmoregulatory actions are considered by some to be the common and primary action of PRL throughout the vertebrates (Hirano 1986). For example, PRL caused several amphibians to move to an aquatic habitat (Warburg 1995). Mudskipper fishes (Perciformes; Gobioidae) are amphibious and euryhaline: they spend the greater parts of their lives out of water, and also maintain osmotic balance in hypotonic fresh water as well as in hypertonic sea water. Therefore, they provide a unique model for studies on osmoregulatory action of PRL under both terrestrial and aquatic conditions, and may be an evolutionary link between terrestrial and aquatic vertebrates for the existence of an extrapituitary PrRP–PRL axis.

Against this background, we thought that the analyses of expression profiles of PrRP and PRL in the various organs during adaptation of the mudskipper to different osmotic environments would give a clue to the relationships between PrRP and PRL. In the present study, we show the tissue distribution of mRNAs for mudskipper PrRP, PRL and GH, as well as the changes in their levels during the terrestrial adaptation and adaptation to different salinities by Northern blotting. In addition, we examined the cellular localization of immunoreactive PrRP and PRL mRNA in the intestine, an osmoregulatory organ, to ascertain the morphological basis for the innervation.

Materials and methods

Animals

Adult mudskippers (*Periophthalmus modestus*) of both sexes weighing 4–6 g were obtained from the estuary of the Fujii River that pours into the Inland Sea of Seto. The fish were kept in the laboratory in tanks of isotonic one-third sea water (22–25 °C) for more than 1 week (Sakamoto *et al.* 2000a). A small plate floated in each tank so that the animals could climb onto this plate *ad libitum*. Freshly excised tissues for RNA extraction were immediately frozen in liquid nitrogen and then stored at –80 °C.

Experimental protocol

The fishes were transferred from one-third sea water to either one-third sea water (control), fresh water (0 parts

per thousand (ppt)), sea water (30 ppt) or to aquaria without water ($n=4-6$ per group). Fishes transferred to one-third sea water, fresh water, or sea water were sampled after 10 h and 1 week. Since previous studies reported that many physiological changes occurred approximately 10 h after water deprivation when fishes lose water to a 20% reduction of initial body mass (Gordon *et al.* 1978, Lee & Ip 1987, Sakamoto *et al.* 2002), the water-deprived fishes were sampled at this time point. This transfer study protocol was repeated three times and representative results are shown.

After quick blood collection by syringe from the hemal arch in the region of the caudal peduncle, the brain, pituitary, liver and gut were immediately removed, frozen in liquid nitrogen and then kept at –80 °C. The plasma was stored at –40 °C after centrifugation. To avoid stress, fish were anaesthetized with tricaine methane sulfonate before handling. All fish were kept, handled and used in accordance with Guidelines for Animal Experimentation of Okayama University.

RNA extraction

Total RNA was extracted from pituitaries by the method of Chomczynski & Sacchi (1987) using an RNA purification kit (Isogen; Wako Chemical, Osaka, Japan). Poly(A)⁺ RNA was obtained from other tissues by oligo(dT)–Sepharose chromatography using a Micro-prep RNA kit (Pharmacia) according to the manufacturer's protocol. Each set of tissue samples was extracted at the same time to avoid procedure variability among the groups. The RNA was quantified by spectrophotometry.

PrRP cDNA probe

First-strand cDNA was reverse-transcribed from brain poly(A)⁺ RNA using the First Strand cDNA Synthesis kit (Pharmacia Biotech, Uppsala, Sweden). Following the manufacturer's protocol, the primer 5'-GACCACGCGTATCGATGTCGACT₁₈-3' was used for reverse transcription. One degenerate sense primer (5'-GA(TC)CC(AG)TTCTGGTA(CT)GTGG-3') designed based on the conserved regions of PrRPs, and an anchor primer (5'-GACCACGCGTATCGATGTCGAC-3') were used to clone the 3' partial region of mudskipper PrRP cDNA.

During PCR, the 50 µl reaction mixture (1 µl first-strand cDNA, 0.4 µM each sense and anchor primer, 0.2 mM nucleotide mix and 5 µl 10 × PCR buffer (final concentration, 10 mM Tris/HCl, pH 8.8, 50 mM KCl, 2.5 mM MgCl₂, 1.5 units Gold-*Taq* DNA polymerase; PE Applied Biosystems, Chiba, Japan)) was subjected to 50 cycles of amplification. After activation of *Taq* polymerase at 95 °C for 10 min, each cycle

consisted of a 1-min denaturation at 96 °C, 30-s primer annealing at 52 °C and 1-min primer extension at 73 °C. The final extension was 7 min at 72 °C.

A PCR-amplified cDNA product of expected size (363 bp) was observed after agarose gel electrophoresis (visualized by ethidium bromide staining). The DNA was extracted from the gels and ligated into pT7 Blue T-Vector (Novagen, Madison, WI, USA). Nucleotide sequencing of both strands was performed using an ABI DNA Sequencer 373 (Perkin-Elmer, Foster, CA, USA). Multiple clones were examined.

This partial sequence was identified as a PrRP cDNA fragment, because the optimized alignment of the corresponding regions of PrRP obtained from teleosts (Fujimoto *et al.* 1998, Moriyama *et al.* 2002, Seale *et al.* 2002) revealed 70% among the sequences, the topologies of the phylogenetic trees of the aligned sequences were in accordance with the known phylogeny of teleosts (Clustal method) and the sequence did not match significantly other sequences deposited in the databases. The partial nucleotide and derived amino acid sequences of mudskipper PrRP are available from the GenBank nucleotide sequence database under accession no. AB089193.

cDNA probes for GH and PRL

First-strand cDNA was reverse-transcribed from pituitary RNA using the First Strand cDNA Synthesis kit with the NotI-d(T)₁₈ primer (Pharmacia). Degenerate sense and antisense primers were synthesized based on the most conserved regions of the sequence of known teleost PRLs or GHs to clone the internal region of mudskipper cDNAs encoding PRL and GH. During PCR of the first-strand cDNA, primers for PRL (5'-GACAA(AG)CT(GT)CACTC(ACT)CTCAG(CTG)-3' (sense) and 5'-GCCCGGCA(ACGT)C(GT)(ACG)AG(GA)AC(CT)TT(CG)AGGAA-3' (antisense)) or GH (5'-CC(ACGT)AT(ACT)GA(CT)AA(AG)CA(CT)GA-3' (sense) and 5'-TC(AGTC)AC(TC)TT(AG)TGCAT(AG)TC-3' (antisense)), and KOD-Dash DNA polymerase (Toyobo, Osaka, Japan), in a 50 µl reaction mixture, were subjected to 30 cycles of amplification. Each cycle consisted of 1 min at 94 °C, 30 s at 54 °C and 90 s at 74 °C. PCR products of the expected size (497 bp for PRL or 314 bp for GH), which correspond to amino acid residues 40–205 of tilapia PRL₁₈₈ or 61–167 of bonito GH (Rand-Weaver *et al.* 1993), were seen after agarose gel electrophoresis. The DNA was extracted from the gels and ligated into pT7 Blue T-Vector (Novagen). Nucleotide sequencing of both strands was performed using an ABI DNA Sequencer 373 (Perkin-Elmer). Multiple clones were examined. These sequences were identified as target cDNAs also based on the alignment of the perciform cDNAs (Rand-Weaver *et al.* 1993). The sequences of PRL and GH are available

from the GenBank nucleotide sequence database under accession nos. AB089194 and AB089195, respectively.

Northern blot analyses

The above mudskipper probes and cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with [α -³²P]dCTP (random priming, BcaBEST DNA labeling kit; Takara, Otsu, Japan). Total RNA (2 µg) of the pituitary or poly(A)⁺ RNA (5 µg) of other tissues were size fractionated through a 1% agarose-formaldehyde gel and transferred onto a nylon membrane (Amersham) by capillary blotting (Sambrook *et al.* 1989). The RNA was covalently attached to the membrane by baking at 80 °C for 2 h and by UV cross-linking. The membranes were hybridized with the probe for PrRP, PRL, GH or GAPDH for 18 h following prehybridization in Perfecthyb hybridization solution (Stratagene) according to the manufacturer's instructions. The membranes were washed in 2 × SSC and 0.1% SDS at 65 °C for 2 × 10 min. They were washed again with 1 × SSC containing 0.1% SDS at 65 °C for 20 min, 0.1 × SSC containing 0.1% SDS for 2 × 20 min at 65 °C, and then rinsed at room temperature. The membranes were exposed to phosphorimaging plate (Fuji imaging plate, Bas III; Fuji Film, Tokyo, Japan). Intensity of the hybridization signals was assessed with an Auto Image Analyser (Bas 2000; Fuji Film). After analysis of one of the messages, the membranes were dehybridized as described by the manufacturer followed by autoradiographies to check that the probes were removed. The membranes were then rehybridized to the other probes sequentially. Serial dilutions of the RNA demonstrated linearity between hybridization signals and serial dilutions (results not shown). mRNA data are represented in arbitrary units normalized to the quantity of GAPDH signals, which were relatively constant, and pooled RNA was used as an internal standard to adjust the variability among the blots. Molecular sizes were estimated relative to migration of a RNA size marker (Toyobo).

PrRP–PRL localization in the gut

The mudskippers were kept in fresh water for 7 days ($n=4$), and the gut was fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffer fixative (pH 7.4) at 4 °C. The anterior part of the intestine, where PrRP and PRL expression was abundant, as shown by Northern blots (results not shown), was dehydrated in ethanol and embedded in paraplast (Monoject; Sherwood Medical, St Louis, MO, USA). In the mudskipper, the oesophagus is connected directly to the proximal intestinal swelling. Two sagittal serial sections (5 µm on a microtome) were mounted

separately on 3-aminopropyltriethoxysilane-coated slides.

Immunocytochemical staining of PrRP was carried out with anti-synthetic C-RFa serum using a Histofine immunostaining kit (Nichirei, Tokyo, Japan) basically as described by Moriyama *et al.* (2002). Anti-synthetic C-RFa serum was diluted 1:5000 with 0.1 M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100. To test the specificity of the immunoreaction, the adjacent sections were incubated with anti-synthetic C-RFa serum that was preabsorbed overnight at 4 °C with excess synthetic C-RFa (1 µg peptide in 1 ml antiserum).

For *in situ* hybridization of PRL mRNA, the fluorescein-labelled oligonucleotide probe against mudskipper PRL (5'-GACACTTGGAGAGCCTGGTCTT TGTC AGTAGGACCTTGCAGATTGGACG(FITC-dT)(FITC-dT)(FITC-dT)-FITC-3') was synthesized commercially (Amersham Pharmacia Biotech, Tokyo, Japan). Hybridization and washes were performed in the dark according to Hyodo & Urano (1991). In brief, following prehybridization for 1 h, hybridization was carried out at 37 °C for 16 h with 500 ng/ml probe dissolved in the hybridization medium containing 20 mM Tris/HCl (pH 7.5), 6 mM EDTA, 0.6 M NaCl, 10% dextran sulfate, 1 × Denhardt's solution, 0.5 mg/ml calf thymus DNA and 40% deionized formamide. The slides were washed at a final stringency of 30% formamide/1 × SSC at 37 °C. Control slides for specificity were hybridized with excess (4000-fold) unlabelled probe. The sections were mounted in Gel/Mount (Biomedica, Foster, CA, USA) and observed using a fluorescence microscope (EFD2 with Hg 100 W light source; Nikon, Tokyo, Japan) equipped with a chilled CCD camera (C5985; 756 × 483 × 8 bit; Hamamatsu Photonics, Hamamatsu, Japan).

Plasma ion analyses

Plasma Na⁺ (an indicator of plasma osmolality) was determined by atomic absorption spectrophotometry (Z5300; Hitachi, Tokyo, Japan).

Statistics

All data are presented as means ± S.E.M. Statistical significance of differences among mean values was tested using an ANOVA followed by the least significant difference test with Statview 4.11 (Abacus Concepts).

Results

Tissue distribution of PrRP, PRL and GH mRNAs

Transcripts of PrRP at about 1.7 and/or 3 kb were abundantly expressed in the brain, liver, gut and ovary

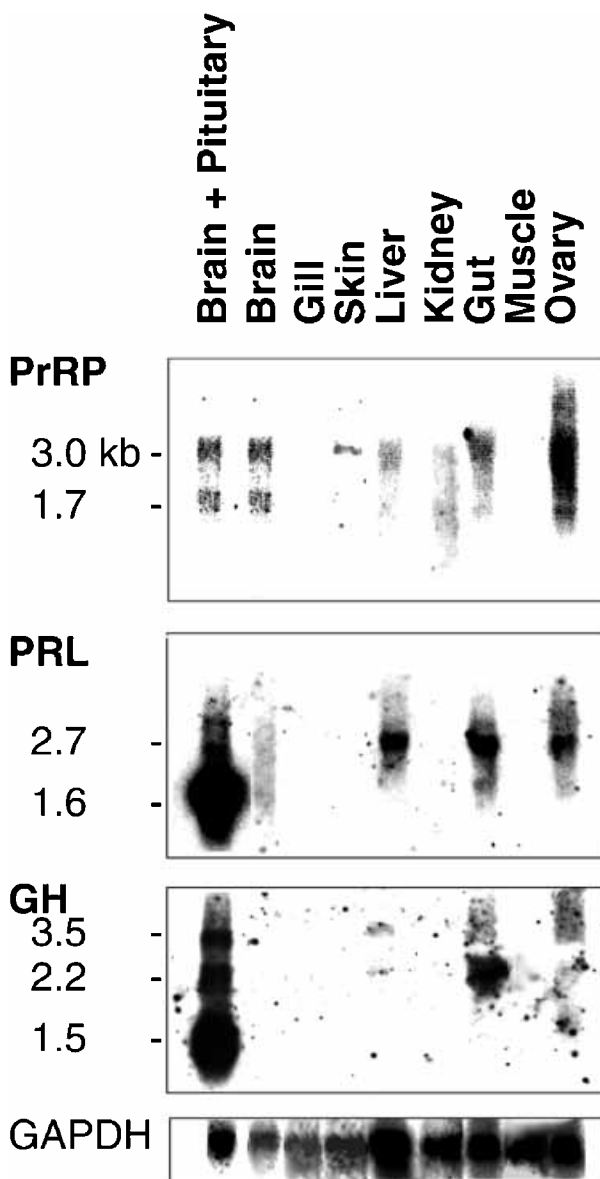


Figure 1 Northern-blot analyses for mudskipper mRNAs of PrRP, PRL and GH. Poly(A)⁺ RNA preparations obtained from the indicated tissues pooled from five fish were electrophoresed, transferred to a filter and then hybridized with [α -³²P]dCTP-labelled cDNA of mudskipper PrRP. The membranes were rehybridized with cDNAs of mudskipper PRL, GH or GAPDH. Sizes of transcripts are indicated.

(Fig. 1), while signals were also observed in the skin and kidney. As for PRL mRNA, the apparent positive signals at about 2.7 kb were detected in the liver, gut and ovary, in addition to the highest level of expression in the pituitary (about 1.6 and 2.7 kb). We could also detect signals for extrapituitary GH transcripts (2.2 and 3.5 kb) in the liver, gut and ovary, in addition to the highest pituitary level (1.5, 2.2 and 3.5 kb). The low levels of the

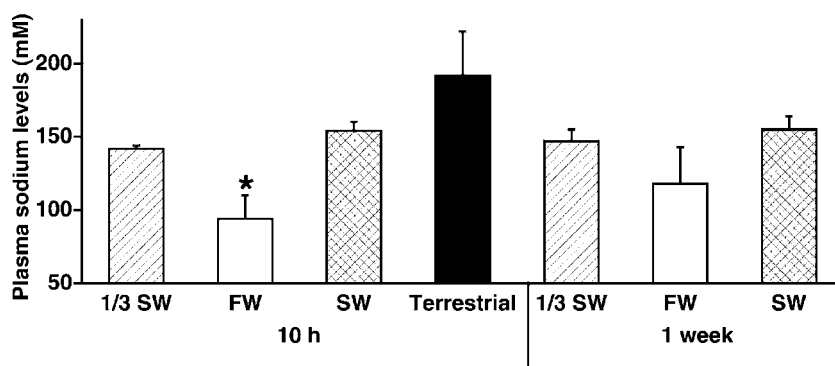


Figure 2 Plasma sodium concentrations in mudskipper under various environmental conditions and control (one-third sea water) for 10 h and 1 week. Values are means \pm S.E.M. ($n=4-6$). For each time period, different letters above bars indicate a significant difference ($P<0.05$ versus control). SW, sea water; FW, fresh water.

extrapituitary signal were not due to blood-cell contamination, as no significant amount of RNA was extracted from blood.

Expression of PrRP, PRL and GH mRNAs during environmental adaptation

When mudskipper were transferred to fresh water, the plasma sodium levels decreased significantly after 10 h ($P<0.05$), and returned to a similar level to that in one-third sea water after 1 week. There was no significant difference in plasma sodium during adaptation to other environments, although a 20% increase was observed in fish kept out of water (Fig. 2).

In the brain (Fig. 3), PrRP mRNA levels increased under terrestrial conditions ($P<0.05$). After 1 week, the brain PrRP mRNA in freshwater fish was higher than that in sea-water fish ($P<0.05$). PRL mRNA levels in the pituitary increased after transfer to fresh water and the value of the 1-week group was significantly different ($P<0.01$). The pituitary PRL mRNA was also elevated under terrestrial conditions ($P<0.05$). On the other hand, GH mRNA levels in the pituitary increased progressively after sea-water transfer and the increase became significant after 1 week ($P<0.05$).

No significant change was seen in the liver mRNA levels either of PrRP, PRL or GH (Fig. 4). In the gut (Fig. 5), mRNA levels of both PrRP and PRL in freshwater fish were higher than those in sea-water fish and the levels of the 1-week group were significantly different ($P<0.05$). The gut GH mRNA increased 1 week after fresh-water transfer ($P<0.05$).

PrRP–PRL localization in the intestine

Figure 6A shows specific immunoreactive PrRP in the anterior intestine of freshwater-adapted mudskipper. Immunoreactive PrRP was observed in the epithelial

cells, especially in the cytoplasm of mucous cells. Little immunoreactivity was found in the controls performed to validate the specificity of the immunoreaction (results not shown).

The localization of the PRL mRNA is shown in Fig. 6B. The hybridization signals were largely located in the PrRP-immunoreactive cells. Some signals were also detected in the lamina propria; however, these signals were differently coloured, not abolished by addition of an excess unlabelled probe, and these seem to be due to the autofluorescence of the blood cells (results not shown). No differences in the distribution of immunoreactive PrRP or signals for PRL mRNA were seen among the different samples.

Discussion

We have reported previously that PrRP promoted specifically PRL transcription and secretion, with the histochemical localization of PrRP neuronal terminals near PRL cells in the pituitaries of teleosts (Wang *et al.* 2000, Moriyama *et al.* 2002, Seale *et al.* 2002, Sakamoto *et al.* 2003b). In the present study, we demonstrated that PrRP and PRL expression are related both topographically and functionally in some organs of the mudskipper, a teleost, suggesting the possible presence of the PrRP–PRL axes in peripheral organs in addition to in the central nervous system.

The abundant expression of PrRP was observed in the brain, liver, gut and ovary in mudskipper, while less-abundant levels were also detected in the skin and kidney. In rat, PrRP mRNA expression was detected in the thyroid gland, trachea, submandibular gland, pancreas, gut, and reproductive organs in addition to the central nervous system. Among human peripheral tissues, the relatively high levels of PrRP mRNA were detected in the pancreas (Fujii *et al.* 1999). Although it is unknown whether the liver of mudskipper is a

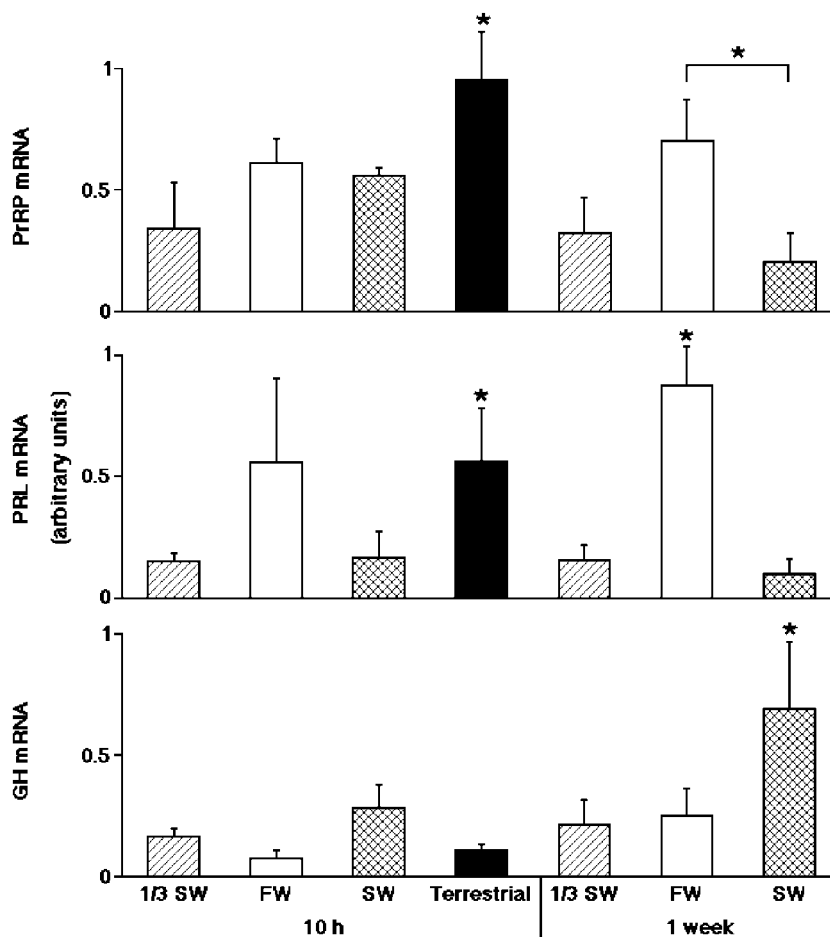


Figure 3 mRNA levels for PrRP in brain and for PRL and GH in pituitary from mudskipper under various environmental conditions and control (one-third sea water) for 10 h and 1 week. mRNA levels were examined by Northern blot (see Fig. 1). Values are means \pm S.E.M. and are expressed as arbitrary units ($n=4-6$). For each time period, different letters above bars indicate a significant difference ($P < 0.05$ versus control). SW, sea water; FW, fresh water.

hepatopancreas, the hybridization band in the liver may be due to the pancreatic tissues.

Corresponding to the distribution of PrRP mRNA in the mudskipper, relatively high expression of extrapituitary PRL mRNA was observed in the liver, gut and ovary. Since such high extrapituitary expression has been reported only in teleosts such as rainbow trout, goldfish and other perciforms (Santos *et al.* 1999, Yang *et al.* 1999, Imaoka *et al.* 2000), production of PRL might be centralized into pituitary during terrestrial tetrapod evolution. The larger size of extrapituitary PRL transcripts may reflect a difference of promoter usage as reported in mammalian lymphocytes and decidua, whose RNA transcripts are larger than the pituitary counterpart (Ben-Jonathan *et al.* 1996). GH gene expression was also detectable in these organs as in salmonid fishes (Yang *et al.* 1999).

During adaptation of the mudskipper to different environments, the changes in mRNA levels of PrRP and PRL paralleled in the brain-pituitary, liver, and gut. PrRP mRNA in the brain and the pituitary PRL mRNA increased under 10 h terrestrial conditions, indicating the activation of the brain-pituitary axis of PrRP-PRL during terrestrial adaptation and perhaps the relative importance of the pituitary PRL in terrestrial vertebrates. The increased plasma levels of sodium/osmolality and cortisol during terrestrial adaptation (Sakamoto *et al.* 2002; Fig. 2) have been known to suppress PRL expression in teleosts (Sakamoto *et al.* 2003a). Under terrestrial conditions, the brain PrRP seems to importantly induce pituitary PRL. The induced pituitary PRL, as circulating PRL, may stimulate a preference for an aquatic habitat as observed in some amphibians. Indeed, when *Xenopus* was kept out of the

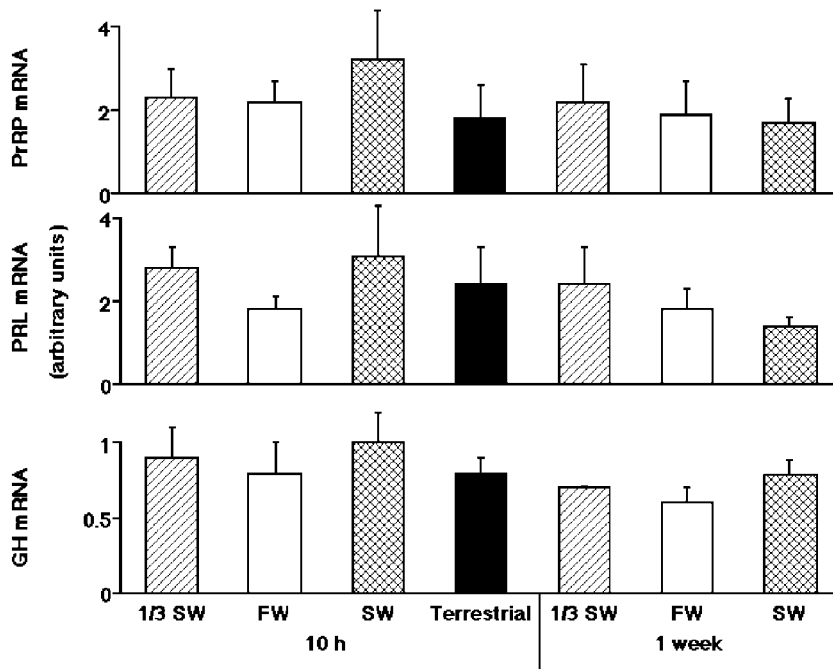


Figure 4 mRNA levels for PrRP, PRL and GH in liver from mudskipper under various environmental conditions and control (one-third sea water) for 10 h and 1 week. mRNA levels were examined by Northern blot (see Fig. 1). The 3.0 kb PrRP mRNA, 2.7 kb PRL mRNA and 3.5 and 2.2 kb GH mRNAs were analyzed, since the nature of other additional mRNAs was uncertain. Values are means \pm S.E.M. and are expressed as arbitrary units ($n=4-6$). For each time period, no significant difference was seen ($P>0.05$). SW, sea water; FW, fresh water.

water PRL was synthesized (Warburg 1995). The brain PrRP and pituitary PRL mRNAs increased after 1 week in fresh water as well, demonstrating that the brain-pituitary axis also operates in fresh water. The pituitary PRL may decrease the integumental permeability in order to maintain the electrolytes in fresh water and the water in terrestrial environment. We have shown that the tissue resistance in the chloride-secreting skin of mudskipper increased during fresh-water adaptation and terrestrial adaptation, although chloride secretion diminished only during fresh-water adaptation (Sakamoto *et al.* 2000b). Actually, the primary action of PRL has been suggested to be the reduction in ion and water permeability of osmoregulatory surfaces without affecting their ion-transporting capacities (Hirano 1986). On the other hand, GH mRNA levels in the pituitary were elevated 1 week after sea-water transfer, consistent with the indirect inhibition of pituitary GH expression by brain PrRP (Sakamoto *et al.* 2003a). A sea-water-adapting action of GH in mudskippers appears to be similar to that reported in several teleosts such as salmonids, tilapia and killifish (Sakamoto *et al.* 1993, 1997, 2001).

Expressions of PrRP and PRL in the gut of freshwater fish were higher than those in sea-water fish after

1 week, although there was no change in fish kept out of water. Therefore, the axis in the gut operates specifically during fresh-water adaptation. In the gut, PrRP and PRL seem to be co-localized in the intestinal epithelia, especially in the mucus cells, contrary to the presence of PrRP mRNA in the lamina propria and the effects of PrRP on motor function in rat intestines (Roland *et al.* 1999, Grabauskas *et al.* 2004). In the intestinal epithelia, PrRP may stimulate PRL expression in an autocrine manner as observed in human decidua (Reis *et al.* 2002). The induction of intestinal PRL in fresh water may have relevant implications. Specifically during fresh-water adaptation, intestinal PRL may participate locally in the epithelial (mucus) cell growth and/or in the inhibition of apoptosis, since the gut mucus cells are numerous in fresh-water fish, but not in sea-water fish (Yamamoto & Hirano 1978). A large number of PRL's actions are associated with cell proliferation and/or apoptosis, and PRL has been shown to induce the hypertrophy of the intestinal mucosa in birds and mammals as well as the mucous cell size and number in fish gills (Bole-Feysot *et al.* 1998, Sakamoto *et al.* 2005). In the intestinal mucus cells of tilapia, PRL receptor mRNA has been clearly observed (Sandra *et al.* 2000). PrRP has also been suggested to promote growth and survival of

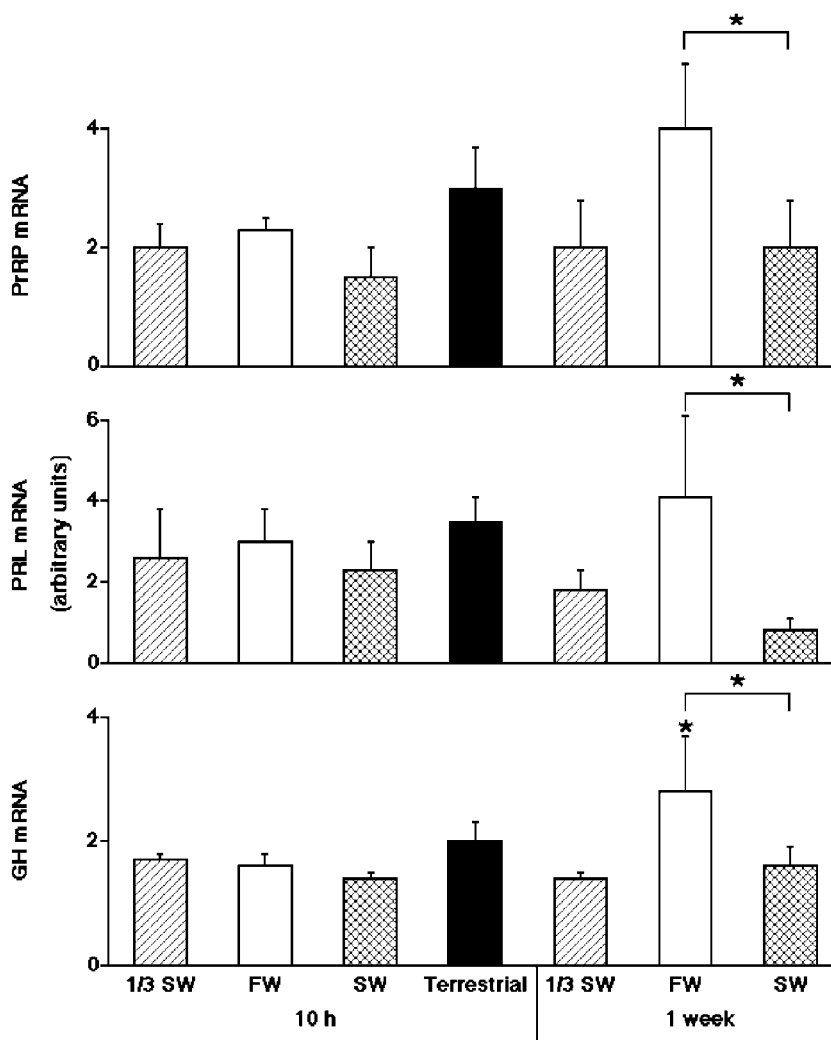


Figure 5 mRNA levels for PrRP, PRL and GH in gut from mudskipper under various environmental conditions and control (one-third sea water) for 10 h and 1 week. mRNA levels were examined by Northern blot (see Fig. 1) and analyzed as described for Fig. 4. Values are means \pm S.E.M. and are expressed as arbitrary units ($n=4-6$). For each time period, different letters above bars indicate a significant difference ($P < 0.05$ versus control). SW, sea water; FW, fresh water.

PRL-producing cells (Duval & Gutierrez-Hartmann 2002), i.e. the mucus cells in the intestine. Furthermore, the increased expression of gut GH in fresh water may also be involved in the growth and differentiation of particular cells.

In the liver, no significant change was seen in PrRP, PRL or GH, suggesting their minor role during adaptation to different environments. The variation among organs may reflect the functional versatility of PrRP–PRL axis (Hirano 1986, Sakamoto *et al.* 2003a). Future studies should determine the effect of PrRP in the control of extrapituitary PRL production using the

neutralizing antibodies or antisense oligonucleotides possibly with *in vitro* preparations.

In conclusion, we have shown that PrRP and PRL are co-expressed in some organs in a teleost during adaptation to different environments, suggesting that PrRP regulates PRL expression at the peripheral organs as well as at the pituitary. The possible presence of PrRP–PRL axes in the peripheral organs adds a new candidate for a specific local modulator of extrapituitary PRL, suggesting an ancient history of this axis prior to the evolution of the hypothalamus–pituitary. PrRP might be an original, fundamental regulator of PRL.

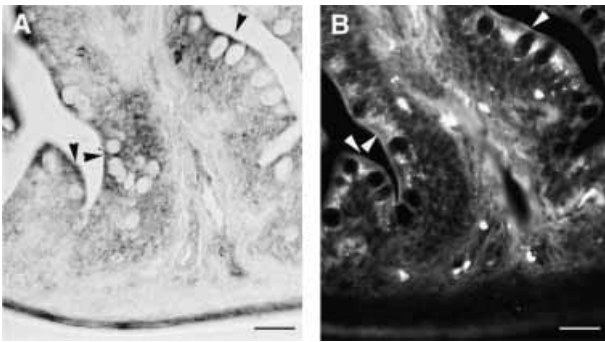


Figure 6 Cellular localization of PrRP-PRL in fresh-water mudskipper intestine. PrRP immunostaining is present in the epithelium, primarily in the cytoplasm of mucus cells (A; arrowhead). PRL mRNA is also expressed in the mucus cells by *in situ* hybridization (B; arrowhead). Scale bars, 10 μ m.

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