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Gene Structure and Seasonal Expression of Carp Fish Prolactin Short Receptor Isoforms

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Abstract The complex adaptive mechanisms that eurythermal fish have evolved in response to the seasonal changes of the environment include the transduction of the physical parameter variations into neuroendocrine signals. Studies in carp (*Cyprinus carpio*) have indicated that prolactin (PRL) and growth hormone (GH) expression is associated with acclimatization, suggesting that the pituitary gland is a relevant physiological node in this adaptive process. Also, the distinctive pattern of expression that carp prolactin receptor (PRLr) protein depicts upon seasonal acclimatization supports the hypothesis that PRL and its receptor clearly are involved in the new homeostatic stage that the eurythermal fish needs to survive during the cyclical changes of its habitat. Here, we characterize the first prolactin receptor gene in a teleost and show that its expression is not associated with alternative promoters, unlike in humans and rodents. Using the regulatory region to direct the transcription of green fluorescent protein (GFP) in zebrafish embryos, we mapped the appearance of this hormone receptor during fish development. This is the first report identifying a fish prolactin receptor gene expressing transcript isoforms encoding for short forms of the protein (45 kDa). These have been found in osmoregulatory tissues of the carp and are regulated in connection with the seasonal acclimatization of the fish. J. Cell. Biochem. 100: 970–980, 2007.

Key words: teleost; prolactin receptor gene; prolactin receptor isoforms; acclimatization; zebrafish development

Prolactin (PRL) is a polypeptide hormone of the growth hormone (GH)/cytokine family, produced by pituitary lactotroph cells, which have over 300 different functions in higher vertebrates [Goffin et al., 2002]. The PRL effects are mediated by the prolactin receptor (PRLr), a member of the cytoquines class I receptor family [Bole-Feysot et al., 1998]. In addition, the hormone signal transduction implies that multiples isoforms of the receptor arise from the alternative splicing of the mRNA transcripts from a single *PRLR* gene. The best-characterized PRLr isoforms are those that differ in the extension of the intracellular domain (ICD) [Clevenger et al., 2003]. In euryhaline fish, PRL is clearly the fresh water-adapting hormone [Pickford and Phillips, 1959; Manzon, 2002]. Since the first cDNA coding for PRLr in fish was cloned [Sandra et al., 1995], only one form of the PRLr has been described in several teleost species and seems to be similar to mammal's long isoform [Manzon, 2002].

The human *PRLR* gene is located on chromosome 5 and is organized in 11 exons [Hu et al., 1999, 2001]. The *PRLR* gene is under the control of a highly complex regulatory system at the transcriptional level. Up to six alternative promoters have been described in humans that control the tissue-specific expression of

Abbreviations used: PRL, prolactin; PRLr, prolactin receptor; *PRLR*, prolactin receptor gene; GFP, green fluorescent protein.

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this gene [Hu et al., 2002]. The human PRLr mRNAs show diverse 5′-UTR, products of the alternative promoter transactivation. These singular first exons are spliced to a common exon 2. The translation start codon ATG is found on exon 3, and most of ICD is codified for exon 10.

In eurythermal fish, acclimatization—the natural, seasonally adaptive response to physical changes in the environment—begins when the fish senses the gradual temperature and photoperiod shifts and transduces the physical parameters into molecular signals that may coordinate the compensatory physiological and cellular responses. These entail summer and winter rearrangements of molecular and cellular functions and involve the modulation of gene expression [Dietz and Somero, 1992; Vera et al., 1993; Kausel et al., 1999; Sarmiento et al., 2000; Fletcher et al., 2001; Alvarez et al., 2004; Pinto et al., 2005]. Similarly, temperature acclimation, even though a distinctive process [Somero and Hochachka, 2002] also demands gene expression regulation to provide the homeostatic state that fish require for survival [Goldspink, 1995; Figueroa et al., 1997; Arends et al., 1998; Gracey et al., 2004; Podrabsky and Somero, 2004].

We addressed the question as to whether, in the carp, the gradual physical external milieu changes have a functional repercussion in the hypophysis and that this gland therefore plays a role in the adaptive response of the eurythermal teleost [Figueroa et al., 1994, 1997; Kausel et al., 1999; San Martin et al., 2004]. Accordingly, we investigated environmentally induced pituitary gland gene expression and found, in the warm-season-acclimatized carp, a high level of PRL mRNAs when compared with the pituitary gland from winter-adapted fish [Figueroa et al., 1994]. In addition, we determined that transcription factor Pit-1, a known regulator of pituitary gland hormones that participates in the control of PRL expression, is transcribed at remarkably higher levels in summer-acclimatized carp [Kausel et al., 1999]. Furthermore, Pit-1 exhibits a concurrent cell and temporal transcription with the hormone [Kausel et al., 1999]. While GH also is induced environmentally in the carp [Figueroa et al., 2005], the expression of somatolactin, a fish hormone belonging to the PRL/GH family, seems not to be associated with the seasonal acclimatization process [Lopez et al., 2001].

To gain greater understanding of the mechanisms by which PRL may play a role as a seasonal-adapting signal hormone, we characterized a full-length carp prolactin receptor cDNA, coding for the long form of the protein resembling that found in mammalian prolactin receptors, and we identified up to three receptor transcript isoforms in different tissues of the teleost [San Martin et al., 2004]. Advancing the idea that PRL may be a signal molecule engaged in the acclimatization process that eurythermal fish undergo, we postulate that the hormone receptor should exhibit distinctive features when compared to those characterized in ectotherms. We determined, therefore, that it was important to study the origin of the multiple transcripts that codify the carp PRLr and to assess the *PRLR* gene organization and the regulatory elements that may be sustaining the differential gene expression observed during the summer–winter environmental cycle.

We identified the first *PRLR* gene from a teleost fish and report that the corresponding transcripts share a unique 5′-UTR, suggesting a divergent regulation of the expression of the carp gene as compared to humans and rodents. In addition, we demonstrate that some of the transcripts of this carp gene that codifies for PRLr with a shorter ICD correspond to alternative splicing isoforms of the mRNA. We also show that the transcriptional activity is switched on in an early developmental stage, interestingly in forebrain and pronephros, as evidenced in zebrafish embryos.

MATERIALS AND METHODS

Animal and Tissues

Male carp weighing 1,000–1,500 g were caught in the Calle–Calle river and maintained in a fixed 3×4 m cage submerged 2 m in an affluent of the same river with temperatures 18–20°C (summer) and 8–10°C (winter). Tissues from summer- and winter-acclimatized fish were handled as described by San Martin et al. [2004].

Cloning a Carp *PRLR* Gene

Carp genomic DNA fragments were amplified by PCR using the Elongase enzyme mix (Invitrogen) and primers derived from the known cPRLr cDNA sequence (GeneBank accession number AY044448) [San Martin et al., 2004]. The sets of primers used for genomic

amplification were: 5′-gacttacctgagagttgtag-3′ and 5′-acagcgccagagttcctcat-3′, 5′-aggaactctggcgctgt-gct-3′ and 5′-ggacactctcacgaacggac-3′, 5′-gtccg-ttcgtgagagtgtcc-3′ and 5′-ctgttgagtccacataacca-3′. The PCR program included a denaturation step at 95°C for 1 min, 35 cycles of 94°C 30 s, 55°C for 30 s, 72°C for 7 min followed by a final elongation step at 72°C for 5 min. The amplification products were cloned into pBluescript SK+ and sequenced.

The 5′ region of the *PRLR* gene was obtained by inverse PCR. Genomic DNA (50 ng) was digested with *Eco*RI and the DNA fragments were circularized in a 20 μl ligation reaction (T4 DNA Ligase, Promega). The self-ligated mixture was purified with Wizard Minicolumns (Promega) and used as template with primers pointing outwards (5′-ctagcgcgatcattaaagcg-3′ and 5′-atggctgcattgaactggct-3′) during the inverse PCR. The amplification product was cloned into the pGEM-T Easy Vector (Promega) and fully sequenced.

Cloning of Carp PRLr 5′ and 3′ mRNA Regions

To clone the 5′ region of the transcripts, 5′ RACE reactions were carried out using the First Choice RLM-RACE Kit (Ambion) with kidney, gills, intestine, and male gonad total RNA as templates and the antisense sequence 5′-gatttttggtccaggaacag-3′ as primer. A nested PCR amplification was carried out using the primer 5′-ggacatctcacgaacggac-3′ and the products were cloned into the pGEM-T Easy Vector (Promega) and sequenced. The 3′ RACE reaction was performed from carp gills total RNA and reverse transcribed with oligo $dT_{(15)}$. The PCR reaction utilized the sense oligonucleotide 5′-tgcctcctgttcctggacca-3′ that anneals with the transmembrane coding region of the transcripts.

Carp Promoter-GFP Plasmid Construction

A fragment of genomic DNA that includes the promoter region of *PRLR* gene and the first exon was amplified by PCR using Elongase enzyme mix (Invitrogen) and the sense 5′-CCATGG-CTCTTGTGCTTTTTCACTAT-3′ and antisense 5′-CCATGGCAGATGATGAGCTGCAAC-3′ primers. The PCR products were A-tailed and cloned into the pGEM-T Easy Vector (Promega). The recombinant plasmids were digested with *Nco*I, and the promoter fragments inserted into the vector p1.0GFP-Luc [Molina et al., 2001]. The constructions containing the *PRLR* gene promoter sequences in frame with the green fluorescent protein (GFP) reporter gene (cPRLR634-1.0GL) were confirmed by sequencing. Similarly, we constructed a vector carrying a 600 bp of zebrafish prolactin receptor gene promoter into p1.0GFP-Luc (zfPRLR600-1.0GL).

Transient GFP Expression in Zebrafish Embryos

Embryo rearing was performed as described by Westerfield [2000]. Eggs were collected, dechorioned by pronase treatment, and microinjected with 5 nl of plasmid solution (50 ng/μl) at the one-cell stage using pulled glass microcapillary pipets and a Narshige microinjector. After microinjection, the embryos were incubated at 28.5°C in small tanks containing Holtfreter's solution. The GFP protein expression was detected in fish embryos using a Leica DC300F digital camera on a Leica MZ12.5 stereomicroscope.

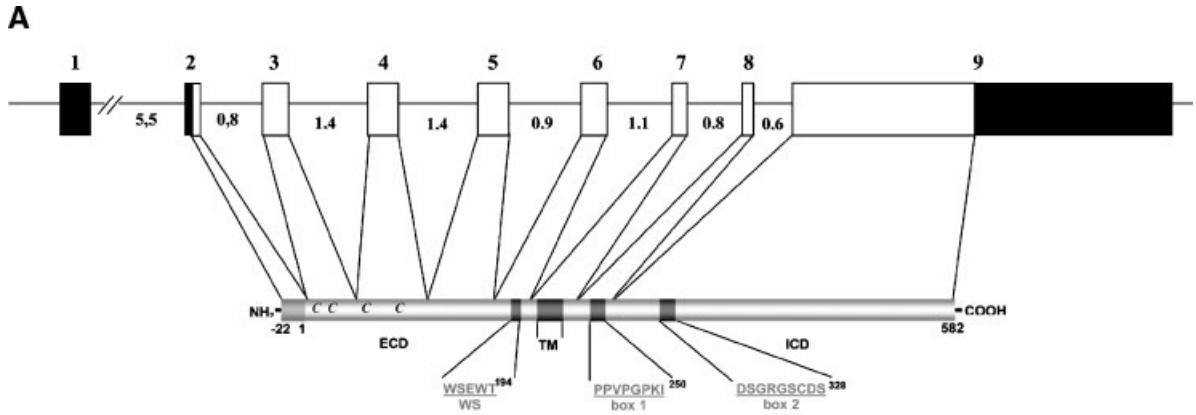
Western Blot

Protein membrane extracts were prepared from kidney, gills, and intestine tissues from summer- and winter-acclimatized carp. Tissues (5 g) were homogenized in 10 vol of homogenization buffer (0.25 M sucrose, 1 mM EGTA, 10 mM Tris pH 7.4) containing the protease inhibitors mix *Complete* (Roche Diagnostic, Germany) and centrifuged at 1,000*g* for 15 min. The supernatant was centrifuged at 20,000*g* for 90 min. All procedures were performed at 4°C. The pellet was suspended in a homogenization buffer and stored at −70°C. For the immune detections, we utilized polyclonal anti-cPRLr sera raised against an extracellular domain (ECD)-derived peptide [San Martin et al., 2004]. Aliquots of protein extracts were solubilized adding 0.1 vol (20% SDS, 50% 2-mercaptoethanol), boiled for 5 min, diluted in 20 vol of buffer 50 mM Tris-Cl pH 7.4, 0.1% Triton X-100, and centrifuged at 20,000*g* for 20 min. The supernatants were fractioned in SDS–PAGE, blotted to Optitran membranes (Schleider and Schuell, Germany), and incubated with the polyclonal antibody. The detection was carried out using the Western Lightning System (Perkin Elmer Life Science).

RESULTS

PRLR Gene in a Teleost Fish

As shown in Figure 1, we identified a carp *PRLR* gene comprising ~16 Kb, which is



B

exon (bp)	intron 3' site		exon (5'- 3')		intron 5' site
		-204		-31	
E1 (174)		ATTTGA		CAGCAG	*gtaagc*
		-30	+1	+64	
E2 (94)	*(not sequenced)*	ATCTAA	ATG	CAGCAG	*gtgatc*
		+65		+197	
E3 (133)	*tcatag*	GTGTCA		AGAAAA	*gtcagt*
		+198		+367	
E4 (170)	*atgcag*	CTCAGA		ATATAG	*gtatgc*
		+368		+534	
E5 (167)	*ccacag*	TTCAAC		TGGGAG	*gtgggt*

exon (bp)	intron 3' site		exon (5'- 3')		intron 5' site
		+535		+676	
E6 (142)	*atgtag*	GAATAT		CTGACT	*gtaagt*
		+677		+773	
E7 (97)	*atgtag*	ATATAC		AAACAG	*gtgcgt*
		+774		+843	
E8 (70)	*attcag*	TGTGAA		CTCAAG	*gtacac*
		+844	+1815		
E9 (2030)	*ttacag*	AGCGGA	TAG		

Fig. 1. Structure of the carp *PRLR* gene. **A**: Organization of the carp *PRLR* gene containing nine exons (rectangles) accounting for 16 Kb of the fish genome. The protein-coding exons for the long form of the receptor are denoted by white rectangles. The intron sizes are indicated but they are not scaled to be compared with the exons. The long form of the carp receptor is correlated with the gene and shows the conserved cysteine residues. Also, the tryptophane-serine (WS) motif in the extracellular domain (ECD) and box 1, 2 in the intracellular domain (ICD). TM, transmembrane region. **B**: The table shows the splicing sites sequences and the exons sizes (bp). The nucleotides are numbered with respect to the ATG translation start codon.

organized in nine exons and eight introns. The exons size varied in a range of 70–174 bp except for exon 9 that contains 2,030 bp. The 5′ and 3′ intron-splicing sites follow the consensus GT and AG sequences. As in mammals, the protein-coding region is restricted from exon 2 to exon 9, and the last exon codes for most of the intracellular carboxy-terminal-domain. The transmembrane domain and the cytoplasmic region, which contains box 1, are encoded in exons 7 and 8, respectively. The ECD of the mature receptor is coded in four exons. The exons 3 and 4 entail the subdomain D1, which includes the conserved cystein residues and glycosylation sites. The subdomain D2 containing WS motif is codified by exons 5 and 6. The 5′-UTR and signal peptide coding region is comprised in exons 1 and 2 (Fig. 1).

The 5′ RACE analyses showed that the 5′-UTR of the cPRLr transcripts were coded by exon 1, in addition to the ATG upstream sequence contained in exon 2. We did not find sequence differences in 5′ region of transcripts amplified from carp kidney, gills, and male gonad RNAs (data not shown).

As depicted in Figure 2, the first nucleotide of the transcripts was adenine in the context GCATTTG that partially matched the consensus initiator (Inr) sequence Py Py A_{+1} N (T/A) Py Py of mammalian cells [Javahery et al., 1994]. Only in kidney were we able to detect a low level of transcripts starting 144 nt downstream of the major start site found in other tissues.

Cis Promoter Elements in Carp Proximal Region *PRLR* Gene

We identified 467-bp upstream of the transcription carp *PRLR* gene start site (Fig. 2). Clearly, it contains a non-classic TATA box. When the whole sequence was searched for transcription factors binding sites, the SP-1, ETS, AP-1, and the tissue-specific MYF, Pit-1, and CDX-1 *cis* promoter elements were recognized.

The Proximal Carp Promoter Region Controls the GFP Expression in Zebrafish Embryos

When the cPRLR634-1.0GL plasmid, containing the carp sequence depicted in Figure 2, was microinjected into zebrafish embryos (Fig. 3), the GFP reporter gene expression occurred in a temporal and tissue-specific form. Accordingly, GFP expression was detected as early as 36 h

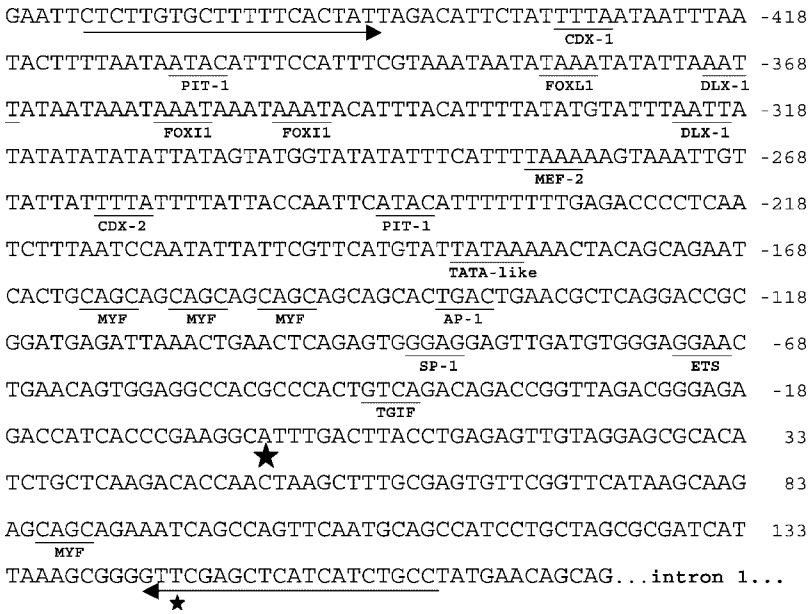


Fig. 2. Proximal promoter region and the first exon of the carp *PRLR* gene. The nucleotides are numbered from the major transcription start site. The stars indicate the transcription start sites obtained from 5′ RACE analysis. The putative binding sites for transcription factors (underlined) were scanned using MatInspector [Quandt et al., 1995]. The arrows below the sequences show the derived primers used for the PCR amplification of this promoter region. This region was utilized in the construction of plasmid cPRLR634-1.0GL for zebrafish embryos microinjections.

post-fertilization (hpf) in forebrain. Although the fluorescent signal was localized exclusively in zebrafish embryo brain at 48 hpf, upon 60 hpf GFP expression also was evidenced in the pronephro embryos. After 72 hpf strong GFP expression was evidenced in the pronephric ducts (Fig. 3). An identical pattern of GFP expression was obtained when a 600-bp zebrafish

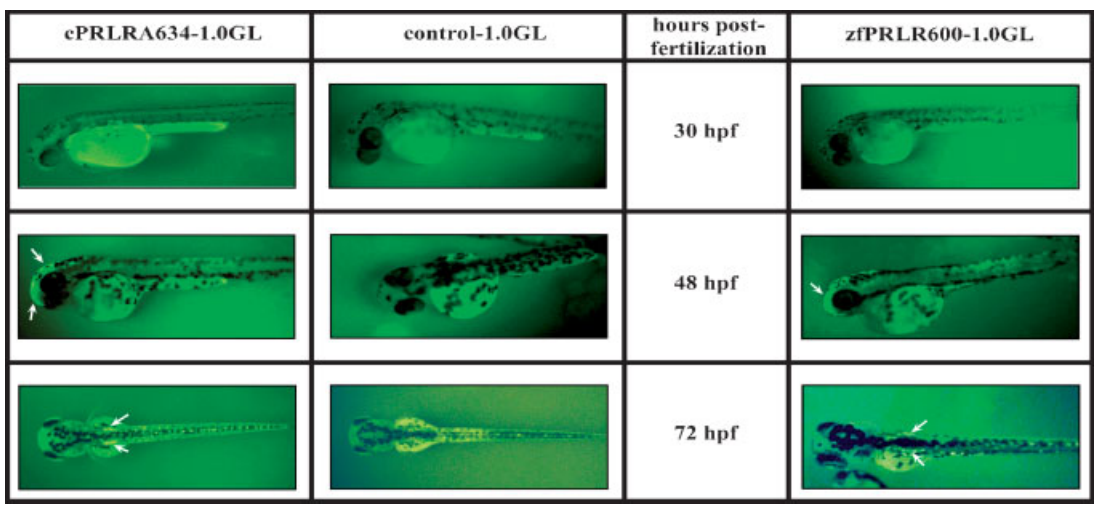


Fig. 3. GFP expression in zebrafish development. The promoter region of carp *PRLR* gene was cloned upstream to the GFP coding region into the plasmid cPRLR634-1.0GL. Similarly, the zebrafish *PRLR* gene promoter was inserted into the plasmid zfPRLR600-1.0GL. Zebrafish embryos were microinjected at one cell stage with the constructs cPRLR634-1.0GL (n = 50), zfPRLR600-1.0GL (n = 45), and matched controls with 1.0GL plasmid (n = 40) and developed at 28.5°C up to 72 h post-fertilization (hpf). The arrows indicate the GFP expression into the embryos.

prolactin receptor promoter was used instead of the carp regulatory sequence, as assayed through zebrafish embryo microinjections (Fig. 3).

Short Prolactin Receptor Isoforms

Because earlier work suggested the expression of tissue-dependent carp prolactin receptor transcripts of different sizes [San Martin et al., 2004], the shorter ones being present in gills, we proceeded with the characterization of these isoforms. Using 3′ RACE and RNA from this tissue (Fig. 4A), we identified a major amplification product corresponding to a carp prolactin receptor cDNA with a shorter ICD. As depicted in Figure 4, this domain contains 71 amino acids compared to the 350 residues comprised in the long protein isoform of the cPRLr previously reported [San Martin et al., 2004]. This isoform identified as cPRLrS1 codes for a mature receptor isoform containing 303 amino acids. The first 293 residues are encoded by the same reading frame comprised within the long cPRLr transcript followed by a distinctive sequence coding for the remaining 10 amino acids (Fig. 4C). In addition, we cloned a minor represented transcript isoform coding for a receptor with an ICD of 118 amino acids (cPRLrS2). This transcript shares the first 331 codons of the long cPRLr mRNA and exhibits a distinctive 3′ sequence encoding 19 C-terminal receptor amino acid residues (Fig. 4C). As shown in Figure 4B, the distinctive 3′ sequences found in cPRLrS1 and cPRLrS2 were not related. Additional amplification products obtained from 3′ RACE (Fig. 4A) were non-homologous to cPRLr mRNA sequence.

When the receptor protein was examined by Western blot using extracts from seasonally adapted carp gills, a predominant signal of ~45 kDa was observed (Fig. 5). This signal also was detected in kidney and intestine proteins. Clearly, the receptor content was higher in gills and intestine from winter-acclimatized carp when compared to protein extracts obtained in the warm season.

When the receptor was assessed in kidney and intestine, additional immune signals representing 70–80 kDa forms were identified. Furthermore, their expression is affected upon seasonal acclimatization (Fig. 5). Intestine proteins from winter-acclimatized carp contain higher levels of the 70–80 kDa receptor forms, while a negligible content was detected in summer-adapted fish. As shown in Figure 5, seasonal acclimatization also affects the qualitative expression of prolactin receptors in kidney. The slight increase of the ~45 kDa signal in the kidney extracts from winter-acclimatized carp observed in Figure 5, may account for the higher content of total protein receptors detected by immunochemistry in kidney sections from cold-season-adapted carp [San Martin et al., 2004].

DISCUSSION

Eurythermal fish have developed physiological compensatory responses to cope with the changing conditions of their habitat, that is, the season-dependent physical features of the environment. The adaptive molecular mechanisms appear to involve, as a central node, the pituitary gland, and, more specifically, the environment-dependent regulation of the expression of PRL [Figueroa et al., 1994, 1997; Kausel et al., 1999; San Martin et al., 2004] and, as shown recently, GH [Figueroa et al., 2005]. Accordingly, PRL is highly expressed in summer-acclimatized carp, while the PRLr transcript levels are remarkably lower during this season in the osmoregulatory tissues of the fish, that is, kidney, intestine, and gills. The hormone-receptor inverse transcription-induction adaptive strategy in response to a hyperosmotic environment has been observed in tilapia gills and intestine. Under this environmental habitat change, PRL decreases while the corresponding tilapia hormone receptors increase [Auperin et al., 1995; Sandra et al., 2001]. A downregulation of PRLr transcription occurs in certain tissues of the carp, but not in the pituitary gland, where cPRLr mRNA levels are maintained when summer- and winter-acclimatized fish are compared [San Martin et al., 2004]. Differences in the cell and temporal expression may be possible through a mechanism involving alternative promoters, as occurs in mammals. Otherwise, through a tissue-specific transcription factors promoter's transactivation.

As our results evidence, the carp *PRLR* gene is smaller than the human gene, which comprises more than 100 kb [Hu et al., 1999, 2002]. A striking difference is observed in the organization of the mRNA 5′-UTR sequence. While in mammals, this same sequence contains an alternative first exon, commonly exons 2 and 3

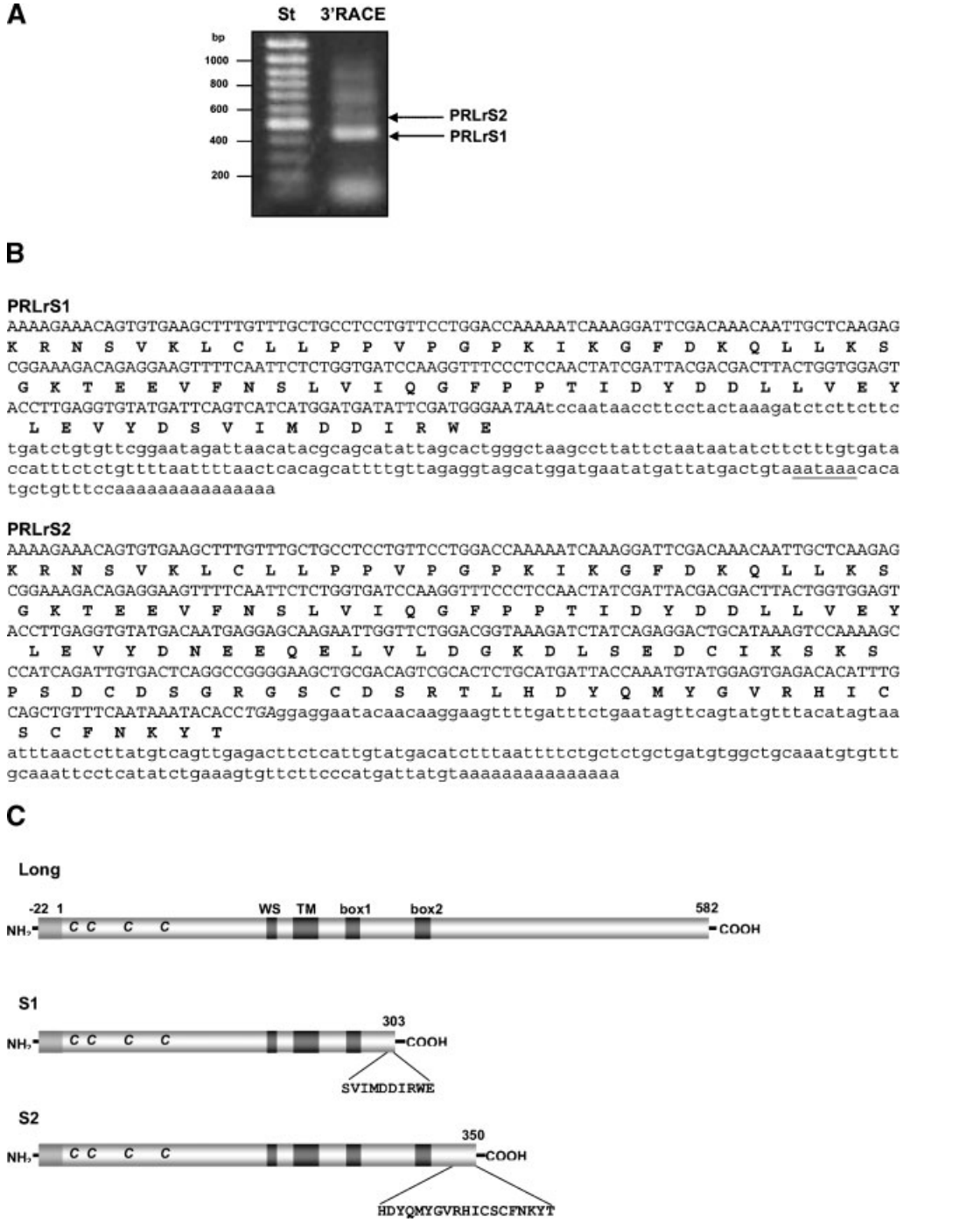


Fig. 4. Short prolactin receptor isoforms in the carp. **A**: 3′ RACE PCR amplification of PRLr transcripts from gills total RNA. The arrows show the amplification products that contain sequences encoding for prolactin receptors isoforms. **B**: Nucleotide sequences of the intracellular coding regions (uppercase) and 3′-UTRs from cloned transcript isoforms PRLrS1 and PRLrS2 (GeneBank accession number DQ086116 and DQ086117, respectively). In PRLrS1, the polyadenylation signal is underlined. Derived amino acids from cDNA sequences are shown below. **C**: Prolactin receptor isoforms derived from the carp *PRLR* gene expression. The distinctive C-terminal amino acid sequences in PRLrS1 and PRLS2 are shown. Amino acids contained in the mature form of the proteins are numbered.

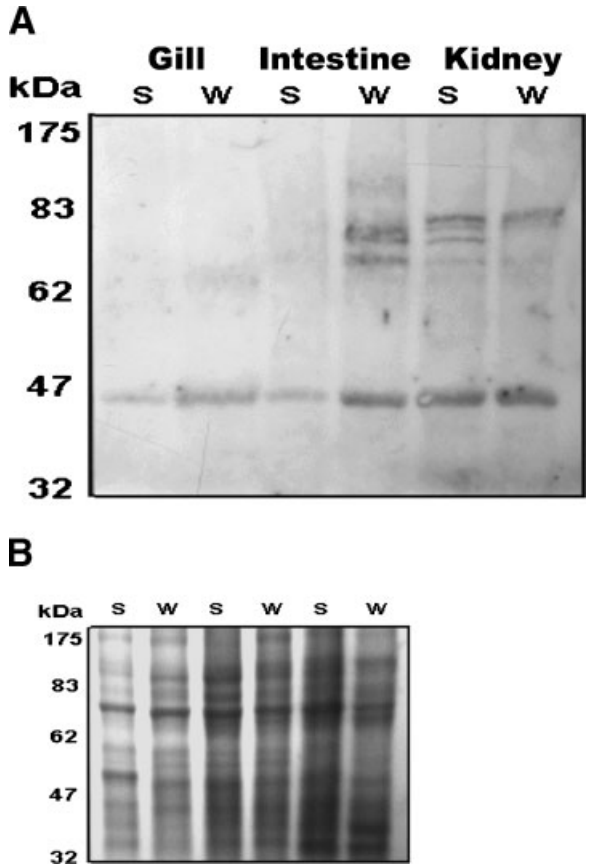


Fig. 5. Immunodetection of carp prolactin receptor isoforms upon seasonal acclimatization. **A**: Membrane protein extracts of gills (10 μg), intestine, and kidney (15 μg) tissues from winter (W)- and summer (S)-adapted carp were fractionated (10% SDS–PAGE), blotted onto nitrocellulose membranes and immunolabeled with an anti-cPRLr polyclonal antibody [San Martin et al., 2004]. Signals for short prolactin receptor isoforms were obtained at ~45 kDa. **B**: Ponceau S staining of proteins blotted onto nitrocellulose membranes.

[Hu et al., 2002], in the carp it is structured by unchanging exons 1 and 2. Although in mammals the *PRLR* gene is clearly controlled by distinctive promoters [Hu et al., 1996, 1997, 2002], in the carp we did not observe differences in the 5′ region of the PRLr transcripts. This could indicate alternative promoter degeneration in this teleost or otherwise genomic rearrangement leading to creation of alternative promoters in mammals [Landry et al., 2003].

It is well established that, in general, mammal prolactin receptor promoters do not contain a consensus TATA box (position −25 to −35). Nevertheless, one of the six human alternative promoters, $hE1_{N5}$, depicts a TATA-like sequence at position −49 [Hu et al., 2002] (Fig. 2). We found a similar situation in carp PRLr promoter where instead of a TATA box a TATA-like sequence was identified (nt −183) (Fig. 2).

When we examined the transactivation capability of the proximal carp promoter, we detected an early onset of GFP expression (36 hpf) in zebrafish development. Santos et al. [2003] has detected the prolactin receptor mRNA as maternal message in sea bream embryos. Our studies in zebrafish embryos (data not shown) are consistent with this observation. Thus, an early requirement for PRL is necessary. The expression of the zebrafish pituitary hormones is initiated in a strict temporal order with similar time courses as in mouse [Kioussi et al., 1999] excepting PRL, which is the first pituitary hormone expressed in zebrafish (22 hpf), whereas it is the last in mouse [Herzog et al., 2003]. Therefore, as a transient onset of PRLr promoter was observed between 36 and 72 hpf (Fig. 3), PRL may be playing a relevant role in brain development in fish larvaes. PRLr expression has been detected by immunohistochemistry in the developing brain of sea bream from 1 dph [Santos et al., 2003]. Besides, PRL may be a relevant modulator of kidney maturation as its expression was present early in the pronephric ducts of the fresh water teleost, in agreement with the osmoregulator role assigned to PRL in fishes [Varsamos et al., 2005] (Fig. 3).

It is well known that PRL, in euryhaline fish, has been implicated mainly as a fresh water-adapting hormone [Manzon, 2002]. The broad expression of the PRLr in fish tissues clearly suggests the multifunctional effects of this hormone, attributable to the differential expression of its receptor isoforms, thus triggering distinctive intracellular signals. Correspondingly, when transfected into PRL-responsive cells, the human PRLr intermediate isoform, while unable to activate the Fyn tyrosine kinase and promote cell proliferation, is capable of mediating cell survival as it occurs in cells expressing the long form of the receptor [Kline et al., 1999; Clevenger et al., 2003]. It also has been shown that the short and long PRLr isoforms can activate Jak2, but the short PRLr does not mediate the PRL activation of the β-casein gene promoter, thus acting as a negative dominant form of the long PRLr [Ali et al., 1992; Lebrun et al., 1995; Hu et al., 2001]. However, in addition to being a negative modulator, the short isoform is able to carry out relevant physiological functions [Telleria et al., 1997;

Bakowska and Morrell, 2003; Binart et al., 2003; Wu et al., 2005].

We have cloned two mRNAs encoding short PRLr isoforms in fish and examined their expression in osmoregulatory tissues of the carp (Figs. 4, 5). These transcripts may arise by alternative splicing from exon 9 to an unknown downstream exon, as occurs with the human short PRLr mRNAs [Hu et al., 2001]. In the sea bream, Northern blot assays have resolved transcripts that could be representing short PRLr mRNA species [Santos et al., 2001; Cavaco et al., 2003]. Also, Weng et al. [1997] have shown by Western blot that the PRLr present in tilapia gills matched to a protein of 42 kDa.

We have found that cPRLr S1 mRNA, which could correspond to the 45 kDa protein detected by Western blots, is the major short receptor form expressed in carp tissues (Fig. 5). The significant protein content of this short form of the receptor is not correlated with the lowest transcript levels observed in Northern blots [San Martin et al., 2004], probably due to the loss of residues from the long proteins that are implicated in ubiquitin-mediated receptor degradation [Ali et al., 2003; Li et al., 2004], thus resulting in a longer half-life for short prolactin receptor form. It is also known that the short PRLr isoforms can differ significantly in their turnover owing to their different C-terminal amino acid extensions [Hu et al., 2001].

The PRLr expression in gills is related to the chloride cells [Weng et al., 1997; Santos et al., 2001] involved in the absorption of Cl^- and Ca^{2+} ions [Perry, 1997]. These cells regulating the excretion of HCO_3^- and H^+ have a relevant role in acid–base homeostasis [Perry et al., 2003]. Because, as we show, carp gills express preferentially the short prolactin receptor, the examination of the role of this isoform in fresh water teleost chloride-control cells is of interest. In kidney, in addition to osmoregulation, immunoregulation also is mediated by PRL [Prunet et al., 2000; Yada et al., 2002; San Martin et al., 2004].

Only one PRLr mRNA has been detected and reported in fish [Sandra et al., 1995; Prunet et al., 2000; Tse et al., 2000; Higashimoto et al., 2001]. Thus, our evidence indicating a seasonal regulation of the content of PRLr isoforms in carp undergoing acclimatization poses new questions about the requirement for short PRLr isoforms in teleost fish and point to a role in the transduction of the PRL signal in the building of the physiologically compensatory response towards the naturally changing environmental condition of the organism's habitat.

REFERENCES

Ali S, Edery M, Pellegrini I, Lesueur L, Paly J, Djiane J, Kelly PA. 1992. The Nb2 form of prolactin receptor is able to activate a milk protein gene promoter. Mol Endocrinol 6:1242–1248.

Ali S, Nouhi Z, Chughtai N, Ali S. 2003. SHP-2 regulates SOCS-1-mediated Janus kinase-2 ubiquitination/degradation downstream of the prolactin receptor. J Biol Chem 278:52021–52031.

Alvarez M, Molina A, Quezada C, Pinto R, Krauskopf M, Vera MI. 2004. Eurythermal fish acclimatization and nucleolar function. A review. J Therm Biol 29:663–667.

Arends RJ, Van der Gaag R, Martens GJ, Wendelaar-Bonga SE, Flik G. 1998. Differential expression of two pro-opiomelanocortin mRNAs during temperature stress in common carp *Cyprinus carpio L.* J Endocrinol 159: 85–91.

Auperin B, Rentier-Delrue F, Martial JA, Prunet P. 1995. Regulation of gill prolactin receptors in tilapia (*Oreochromis niloticus*) after a change in salinity or hypophysectomy. J Endocrinol 145:213–220.

Bakowska JC, Morrell JI. 2003. The distribution of mRNA for the short form of the prolactin receptor in the forebrain of the female rat. Brain Res Mol Brain Res 116: 50–58.

Binart N, Imbert-Bollore P, Baran N, Viglietta C, Kelly PA. 2003. A short form of the prolactin (PRL) receptor is able to rescue mammopoiesis in heterozygous PRL receptor mice. Mol Endocrinol 17:1066–1074.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. 1998. Prolactin PRL and its receptor, actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19:225–268.

Cavaco JE, Santos CR, Ingleton PM, Canario AV, Power DM. 2003. Quantification of prolactin PRL and PRL receptor messenger RNA in gilthead seabream (*Sparus aurata*) after treatment with estradiol-17beta. Biol Reprod 68:588–594.

Clevenger CV, Furth PA, Hankinson SE, Schuler LA. 2003. The role of prolactin in mammary carcinoma. Endocr Rev 24:1–27.

Dietz TJ, Somero GN. 1992. The threshold induction temperature of the 90-kda heat shock protein is subject to acclimatization in eurythermal goby fishes genus gillichthys. Proc Natl Acad Sci USA 89:3389–3393.

Figueroa J, Molina A, Alvarez M, Villanueva J, Reyes A, Leon G, Krauskopf M. 1994. Prolactin gene expression and changes of prolactin pituitary level during the seasonal acclimatization of the carp. Comp Biochem Physiol: Biochem Mol Biol 108:551–560.

Figueroa J, Reyes A, Rios M, Vera MI, Krauskopf M. 1997. Effect of temperature and photoperiod on prolactin transcription in *Cyprinus carpio*. Zool Sci 14:353–357.

Figueroa J, San Martin R, Flores C, Grothusen H, Kausel G. 2005. Seasonal modulation of growth hormone mRNA and protein levels in carp pituitary: Evidence for two expressed genes. J Comp Physiol B 175:185–192.

Fletcher GL, Hew CL, Davies PL. 2001. Antifreeze proteins of teleost fishes. Ann Rev Physiol 63:359–390.

Goffin V, Binart N, Touraine P, Kelly PA. 2002. Prolactin: The new biology of an old hormone. Ann Rev Physiol 64: 47–67.

Goldspink GJ. 1995. Adaptation of fish to different environmental temperature by qualitative and quantitative changes in gene expression. J Therm Biol 20:167–174.

Gracey AY, Fraser EJ, Li W, Fang Y, Taylor RR, Rogers J, Brass A, Cossins AR. 2004. Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. Proc Natl Acad Sci USA 101: 16970–16975.

Herzog W, Zeng X, Lele Z, Sonntag C, Ting JW, Chang CY, Hammerschmidt M. 2003. Adenohypophysis formation in the zebrafish and its dependence on sonic hedgehog. Dev Biol 254:36–49.

Higashimoto Y, Nakao N, Ohkubo T, Tanaka M, Nakashima K. 2001. Structure and tissue distribution of prolactin receptor mRNA in Japanese flounder *Paralichtys olivaceus*, conserved and preferential expression in osmoregulatory organs. Gen Comp Endocrinol 123: 170–179.

Hu ZZ, Zhuang L, Dufau ML. 1996. Multiple and tissue-specific promoter control of gonadal and non-gonadal prolactin receptor gene expression. J Biol Chem 271: 10242–10246.

Hu ZZ, Zhuang L, Guan X, Meng J, Dufau ML. 1997. Steroidogenic factor-1 is an essential transcriptional activator for gonad-specific expression of promoter I of the rat prolactin receptor gene. J Biol Chem 272:14263–14271.

Hu ZZ, Zhuang L, Meng J, Leondires M, Dufau ML. 1999. The human prolactin receptor gene structure and alternative promoter utilization, the generic promoter hP_{III} and a novel human promoter hP_N. J Clin Endocr Metab 84:1153–1156.

Hu ZZ, Meng J, Dufau ML. 2001. Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. J Biol Chem 276:41086–41094.

Hu ZZ, Zhuang L, Meng J, Tsai-Morris CH, Dufau ML. 2002. Complex 5′ genomic structure of the human prolactin receptor, multiple alternative exons 1 and promoter utilization. Endocrinology 143:2139–2142.

Javahery R, Khachi A, Lo K, Zenzie-Gregory B, Smale ST. 1994. DNA sequence requirements for transcriptional initiator activity in mammalian cells. Mol Cell Biol 14: 116–127.

Kausel G, Vera MI, San Martín R, Figueroa J, Molina A, Muller M, Martial J, Krauskopf M. 1999. Transcription factor Pit-I expression is modulated upon seasonal acclimatization of eurythermal ectotherms, identification of two pit-I genes in the carp. J Cell Biochem 75:598–609.

Kioussi C, Carriere C, Rosenfeld MG. 1999. A model for the development of the hypothalamic-pituitary axis, transcribing the hypophysis. Mech Develop 81:23–35.

Kline JB, Roehrs H, Clevenger CV. 1999. Functional characterization of the intermediate isoform of the human prolactin receptor. J Biol Chem 274:35461–35468.

Landry JR, Mager DL, Wilhelm BT. 2003. Complex controls: The role of alternative promoters in mammalian genomes. Trends Genet 19:640–648.

Lebrun JJ, Ali S, Ullrich A, Kelly PA. 1995. Proline-rich sequence-mediated Jak2 association to the prolactin receptor is required but not sufficient for signal transduction. J Biol Chem 270:10664–10670.

Li Y, Kumar KG, Tang W, Spiegelman VS, Fuchs SY. 2004. Negative regulation of prolactin receptor stability and signaling mediated by SCFbeta-TrCP E_3 ubiquitin ligase. Mol Cell Biol 24:4038–4048.

Lopez M, Figueroa J, Kausel G, Vera MI, Krauskopf M. 2001. In situ hybridization of somatolactin transcripts in the pituitary glands from acclimatized carp (*Cyprinus carpio*). Biol Res 34:7–13.

Manzon LA. 2002. The role of prolactin in fish osmoregulation, a review. Gen Comp Endocrinol 125:291–310.

Molina A, Di Martino E, Martial JA, Muller M. 2001. Heat shock stimulation of a tilapia heat shock protein 70 promoter is mediated by a distal element. Biochem J 356: 353–359.

Perry SF. 1997. The chloride cell, structure and function in the gill of freshwater fishes. Ann Rev Physiol 59:325–347.

Perry SF, Shahsavarani A, Georgalis T, Bayaa M, Furimsky M, Thomas SLY. 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes. Their role in ionic and acid-base regulation. J Exp Zool 300A:53–62.

Pickford GE, Phillips JG. 1959. Prolactin, a factor in promoting survival of hypophysectomized killifish in fresh water. Science 130:454–455.

Pinto R, Ivaldi C, Reyes M, Doyen C, Mietton F, Mongelard F, Alvarez M, Molina A, Dimitrov S, Krauskopf M, Vera MI, Bouvet P. 2005. Seasonal environmental changes regulate the expression of the histone variant $macroH_2A$ in an eurythermal fish. FEBS Lett 579:5553–5558.

Podrabsky JE, Somero GN. 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. J Exp Biol 207:2237–2254.

Prunet P, Sandra O, Le Rouzic P, Marchand O, Laudet V. 2000. Molecular characterization of the prolactin receptor in two fish species, tilapia *Oreochromis niloticus* and rainbow trout, *Oncorhynchus mykiss*, a comparative approach. Can J Physiol Pharmacol 78:1086–1096.

Quandt K, Frech K, Karas H, Wingender E, Werner T. 1995. MatInd and MatInspector, new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 23:4878–4884.

San Martin R, Caceres P, Azocar R, Alvarez M, Molina A, Vera MI, Krauskopf M. 2004. Seasonal environmental changes modulate the prolactin receptor expression in an eurythermal fish. J Cell Biochem 92:42–52.

Sandra O, Sohm F, De Luze A, Prunet P, Edery M, Kelly PA. 1995. Expression cloning of a cDNA encoding a fish prolactin receptor. Proc Natl Acad Sci USA 92:6037–6041.

Sandra O, Le Rouzic P, Rentier-Delrue F, Prunet P. 2001. Transfer of tilapia (*Oreochromis niloticus*) to a hyperosmotic environment is associated with sustained expression of prolactin receptor in intestine, gill and kidney. Gen Comp Endocrinol 123:295–307.

Santos CR, Ingleton PM, Cavaco JE, Kelly PA, Edery M, Power DM. 2001. Cloning, characterization, and tissue distribution of prolactin receptor in the sea bream *Sparus aurata*. Gen Comp Endocrinol 121:32–47.

Santos CR, Cavaco JE, Ingleton PM, Power DM. 2003. Developmental ontogeny of prolactin and prolactin receptor in the sea bream *Sparus aurata*. Gen Comp Endocrinol 132:304–314.

Sarmiento J, Leal S, Quezada C, Kausel G, Figueroa L, Vera MI, Krauskopf M. 2000. Environmental acclimatization of the carp modulates the transcription of β-actin. J Cell Biochem 80:223–228.

Somero GN, Hochachka PW. 2002. Biochemical adaptation, mechanism and process in physiological evolution. New York: Oxford University Press.

Telleria CM, Parmer TG, Zhong L, Clarke DL, Albarracin CT, Duan WR, Linzer DI, Gibori G. 1997. The different forms of the prolactin receptor in the rat corpus luteum, developmental expression and hormonal regulation in pregnancy. Endocrinology 138:4812–4820.

Tse DL, Chow BK, Chan CB, Lee LT, Cheng CH. 2000. Molecular cloning and expression studies of a prolactin receptor in goldfish *Carassius auratus*. Life Sci 66:593–605.

Varsamos S, Nebel C, Charmantier G. 2005. Ontogeny of osmoregulation in postembryonic fish: A review. Comp Biochem Physiol A Mol Physiol 141:401–429.

Vera MI, Norambuena L, Alvarez M, Figueroa J, Molina A, León G, Krauskopf M. 1993. Reprogramming of nucleolar gene expresión during the acclimatization of the carp. Cell Mol Biol Res 39:665–674.

Weng CF, Lee TH, Hwang PP. 1997. Immune localization of prolactin receptor in the mitochondria-rich cells of the euryhaline teleost *Oreochromis mossambicus* gill. FEBS Lett 405:91–94.

Westerfield M. 2000. The zebrafish book. A guide for the laboratory use of zebrafish *Danio rerio*. Eugene: University of Oregon Press.

Wu W, Ginsburg E, Vonderhaar BK, Walker AM. 2005. S179D prolactin increases vitamin D receptor and p21 through up-regulation of short 1b prolactin receptor in human prostate cancer cells. Cancer Res 15:7509–7515.

Yada T, Uchida K, Kajimura S, Azuma T, Hirano T, Grau EG. 2002. Immunomodulatory effects of prolactin and growth hormone in the tilapia, *Oreochromis mossambicus*. J Endocrinol 173:483–492.