Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line

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Abstract

In vertebrates, gonadotropins (GTHs) (FSH and LH) are two circulating pituitary glycoprotein hormones that play a major role in the regulation of gonadal functions, including gonadal cell proliferation/differentiation and steroidogenesis. In mammals, it is well known that their biological effects are mediated by highly specific membrane-bound receptors expressed preferentially on the somatic cells of the gonads. However, in fish, binding and functional studies have shown that cross-reactivity may occur in GTH receptors depending on the species. To understand the molecular mechanisms involved in GTH actions, functional characterization of trout GTH receptors and their gonadal gene expression pattern has been carried out. The present study describes the presence of two distinct GTH receptors in trout showing similarities with those of higher vertebrates but also differences in their

structural determinants. *In vitro* functional studies demonstrate that rtLH specifically activates its cognate receptor ($EC_{50} = 117$ ng/ml), whereas purified rainbow trout FSH (rtFSH) activates FSHR but also LHR at supraphysiological doses ($EC_{50} = 38$ vs 598 ng/ml for FSHR and LHR respectively). The high doses of rtFSH required to activate LHR put into question the physiological relevance of this interaction. The use of heterologous chinook GTHs confirms the strong preference of each hormone for its cognate receptor. The gonadal expression pattern of the GTH receptor genes suggests that FSH may play an important role in regulating gonadal functions, not only at the early stages but also at the final stages of the male and female reproductive cycles, in addition to the LH pathway.

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Introduction

The control of the gonadal functions by two plasma heterodimeric glycoproteins (known as gonadotropins (GTHs) and secreted from the gonadotrophs in the anterior pituitary) is a general model in vertebrate reproduction. In fish, two distinct GTH-I and GTH-II have been purified, and the cDNA of the corresponding specific subunits cloned from several species including salmon (Trinh *et al.* 1986, Suzuki *et al.* 1988, Sekine *et al.* 1989, Swanson *et al.* 1989). Based on their molecular structures and physiological effects, a new nomenclature has emerged and fish GTH-I and GTH-II are now referred to as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) respectively.

In fish, GTHs are differentially secreted in the plasma during the reproductive cycle suggesting that they have specific functions (Prat *et al.* 1996, Breton *et al.* 1998, Gomez *et al.* 1999, Sohn *et al.* 1999). FSH may play a determinant role in regulating early gametogenesis. In females, the specific role of FSH has been described with respect to the recruitment of oocytes into the secondary (vitellogenic) growth phase as well as in vitellogenin uptake (Tyler *et al.* 1991, 1997). In males, FSH induced an active spermatogonial proliferation in testicular

explants cultured *in vitro* (Remacle 1976, Loir 1999) and has recently been shown to be involved in Sertoli cell proliferation (Schulz *et al.* 2005). In contrast, LH is the major regulating factor of late gametogenesis during oocyte maturation and ovulation (Jalabert 1976). In salmonids, LH alone stimulates production of the maturation-inducing steroid in the granulosa cells (17,20 β -dihydroxy-4-pregnen-3-one), thus inducing germinal vesicle breakdown (Planas *et al.* 2000).

In addition, *in vitro* studies highlighted the ability of both hormones to stimulate steroid production in both ovaries and testis, although their potency differs and is mainly related to gonadal maturation (Swanson *et al.* 1991, Planas & Swanson 1995).

As in other vertebrates, once released in the plasma, GTHs bind to membrane-bound receptors expressed on the somatic cells of the gonads. The binding of LH to high-affinity receptors was first demonstrated in the salmonid ovary (Salmon *et al.* 1984, Breton *et al.* 1986, Kanamori *et al.* 1987) and the trout testis (Le Gac *et al.* 1988). Evidence for two distinct binding activities in the fish ovary originated from other binding studies (Yan *et al.* 1992, Miwa *et al.* 1994). In the salmon ovary, ligand-binding assays on gonadal tissue sections followed by autoradiography localized a type I receptor (presumably FSHR) on both thecal

and granulosa cells, whereas a type II receptor (presumably LHR) was identified on granulosa cells only. In the testis, type I and type II receptors were localized on cells lining the tubules and on interstitial cells respectively (Miwa *et al.* 1994). Subsequently, the presence of two distinct GTH receptors was confirmed by the molecular cloning of two different cDNA in several fish species: salmon (Oba *et al.* 1999a,b, Maugars & Schmitz 2006), catfish (Bogerd *et al.* 2001, Kumar *et al.* 2001a,b, Vischer & Bogerd 2003), and zebrafish (Kwok *et al.* 2005, So *et al.* 2005). These membrane receptors belong to the G-protein-coupled receptor (GPCR) superfamily and, in particular, to the subfamily of glycoprotein hormone receptors, characterized by a larger extracellular N-terminal domain, a seven helical transmembrane region, and a short intracellular tail.

Although the overall structure is similar to that described in all other vertebrate species, including reptiles, birds, and mammals, marked differences exist with regard to the hormonal specificity of these receptors toward the fish GTHs. Binding studies with highly purified fish GTHs showed cross-reactivity. In coho salmon, type I receptor (FSHR) was able to bind preferentially to FSH and to a lesser extent to LH (Miwa *et al.* 1994). In carp, purified GTH receptors bound preferentially to their cognate GTH but a moderate overlapping recognition was described (Basu & Bhattacharya 2002). Depending on the species, a promiscuous activation of one or the other fish GTH receptors was also reported in functional studies using mammalian cell lines expressing fish receptors. In African catfish, recombinant cFSH and cLH activated FSHR with a similar biopotency (Bogerd *et al.* 2001, Vischer & Bogerd 2003, Vischer *et al.* 2003), whereas in amago salmon, only FSH was able to activate FSHR (Oba *et al.* 1999a). However, hormonal specificity may also depend on the origin of the GTHs. In zebrafish, bovine FSH activated FSHR and LHR, whereas bovine LH specifically activated LHR (Kwok *et al.* 2005). In contrast, recombinant zebrafish FSH stimulated only FSHR, whereas recombinant LH stimulated both FSHR and LHR (So *et al.* 2005).

In summary, data from the binding and functional studies do not allow one to draw a general conclusion on the responsiveness of the piscine receptors to GTHs, even in salmonids, and further studies from other fish species or strains are worthwhile.

Besides the functional characterization, our knowledge of the expression pattern of the GTH receptor genes is scarce and limited to a few fish species including catfish (Kumar *et al.* 2001a,b), zebrafish (Kwok *et al.* 2005), tilapia (Oba *et al.* 2001), and salmonids (Campbell *et al.* 2006, Kusakabe *et al.* 2006).

In the present study, we report the isolation of two distinct cDNA encoding rainbow trout FSH and LH receptors. Analysis of the amino acid sequences of the fish GTH receptors shows a similar, but not identical, structure to that found in other vertebrates. The expression patterns of the corresponding transcripts have been determined during the male and female reproductive cycles. Distinct temporal expression patterns have been observed in female and male trout. The functional specificity of the trout GTH receptors was studied in transient transfected heterologous cells using

purified rainbow trout GTHs (rtFSH and rtLH). Although an rtFSH-induced responsiveness of LHR was observed at supraphysiological doses, we demonstrated that rtLH and rtFSH activate their cognate receptor preferentially.

Materials and Methods

Animal and tissue collection

Male and female rainbow trout (*Oncorhynchus mykiss*) from a fall spawning strain were bred during their first reproductive season at the INRA/PEIMA fish farm (Sizun, France) and held under natural photoperiod and temperature. Monthly sampling was carried out to collect gonads at different gonadal maturation stages. Female gonadal maturation stages were determined as described previously (Jalabert 1976). Trout were anesthetized in 2-phenoxyethanol (0.03% v/v) and ovulation was checked every 3 days. A sample of ovulated females was manually stripped, and kept for 5–15 days after detection of ovulation. Fish were killed by cervical transection and gonads were dissected out of the body cavity under sterile conditions. A piece of the gonads was fixed in Bouin's solution and the rest frozen in liquid nitrogen and stored at -70°C , until RNA extraction. For the two full-grown oocyte stages (end of vitellogenesis and maturation), the ovary was deyolked as described previously (Garczynski & Goetz 1997). The stage of male gonadal maturation was determined by histological analysis after Regaud's hematoxylin/orange G staining (Gabe 1968). Male gonadal stages were determined as described previously (Billard & Escaffre 1975). Gonadal stage I corresponds to immature male, stage II to active spermatogonial proliferation, stage III to meiosis onset, stage VI to full spermiogenesis, stage VIII to spermiation, and finally stage IX to post-spermiation.

Cloning of GTH receptor cDNA

To isolate full-length cDNA encoding GTH receptors in trout, known amino acid sequences, corresponding to glycoprotein hormone receptors, were aligned. Two conserved motifs spanning the third extracellular loop (KVSICLP) and the seventh transmembrane α -helix (PFLYAI) of these GPCR were chosen to design the forward el2 and reverse el3 degenerated primers. RT-PCR was carried out from testis cDNA and a 352 bp DNA fragment, called the FSHR probe in the present study, was isolated and sequenced. This probe was used to screen, as described previously (Sambroni *et al.* 2001), a stage III–IV testicular cDNA library, constructed in λ ZAP II vector (Stratagene, La Jolla, CA, USA). A single positive clone named FSHR B8 (1337 bp) was obtained with the FSHR probe but was lacking the 5' extremity of the open reading frame. To complete the 5' end of the open reading frame, a 5' RACE-PCR was performed using the rapid amplification of cDNA ends, version 2.0 and the high fidelity elongase (GIBCO BRL Life Technologies).

Briefly, 1 µg total RNA was reverse transcribed with a specific primer Asn172 (Table 1) in order to synthesize the first strand cDNA. After removal of RNA template and purification of the first strand cDNA, an oligo-dC tail was added. The tailed cDNA was amplified with the AAP and Asn136 primers. This allowed us to add 1050 bp upstream from the 5' end of the FSHR B8 clone. Completion of the open reading frame was carried out by RT-PCR with the RI-1 forward primer design from the 5' untranslated region of the amago salmon sGTH-RI cDNA (AB030012) and Asn2-specific reverse primer.

For LHR cDNA molecular cloning, a partial cDNA fragment was first obtained by RT-PCR from reverse-transcribed testicular RNA (stage II) with RII-2 and Asn519 primers (Table 1) designed according to the amago salmon (*Oncorhynchus rhodurus*) sGTH-R cDNA (AB030005). The PCR product (440 bp) was cloned into the pCR2.1TOPO cloning vector (Invitrogen) and is called the LHR probe in the present study. A full-length cDNA (clone 2b-1) was isolated by screening the rainbow trout testicular cDNA library with the LHR probe as described above.

The nucleotide sequences of the cDNA encoding either the FSHR (2783 bp) or the LHR (2756 bp) have been submitted to GenBank under the accession numbers AF439405 and AF439404 respectively.

RNA extraction and reverse transcription

Gonadal tissue was homogenized in Trizol reagent (Invitrogen) at a ratio of 1 ml/100 mg tissue. Total RNA was extracted according to the manufacturer's instructions and resuspended in nuclease-free water. Two micrograms of total RNA were reverse transcribed in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV; Promega) according to the manufacturer's

instructions. Reverse transcription of the total RNA was carried out in the presence of either oligo-dT₁₈ (0.5 µg/µg total RNA) for the molecular cloning of the GTH receptor cDNA or in the presence of random hexamers (0.5 µg/µg total RNA) for the quantitative real-time PCR. The reverse transcription reaction was performed in a total volume of 25 µl, at 37 °C for 1 h and 15 min, followed by a 15-min incubation at 70 °C. Control reactions were run without MMLV reverse transcriptase and used as negative controls in the quantitative real-time PCR study.

Quantitative real-time PCR

Quantitative real-time RT-PCR was performed using an i-Cycler iQ (Bio-Rad). The primers used in the PCRs were 28SFw1/28SRv1, RFSHICF2/RFSHICR2, and RLHICF2/RLHICR2 to amplify the 28S rRNA (reference gene), FSHR, and LHR transcripts respectively (Table 1). At least one of the specific PCR primers was designed on an exon/intron boundary of the FSHR and LHR genes. Reverse transcription products were diluted to 1:40 (or 1:2000 for 28S rRNA) and 5 µl were used for each real-time PCR. PCRs were carried out using the qPCR Mastermix Plus for SYBR Green I kit (Eurogentec, Angers, France), according to the manufacturer's instructions. Briefly, a 20 µl reaction mix was set up with 5 µl reverse-transcribed RNA sample, 5 µl appropriate primers (2.4 µM each), and 10 µl 2× reaction buffer. Thermal cycling was conducted at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was analyzed in duplicate. A pool of testicular reverse-transcribed RNA originating from stage II testis was serially half diluted and used as standard curve to check the linearity of the amplification and to calculate primer set efficiency. Primers

Table 1 Primers used in PCR experiments

Primers	Sequences
e12	5'-AAGGTSAGCATTGCTSCC-3'
e13	5'-AATMGCCTATAGCAAAGG-3'
Asn172	5'-GAAGCTCGTGAAGATGAGGATGGC-3'
AAP	5'-GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGIIIG-3'
Asn136	5'-CATGCGCGTTTCAGCACTGGC-3'
RI-1	5'-GGTGCTGGGACGAGAAAAAGACA-3'
Asn2	5'-CGGCCCTCTGCTCCTTTGAA-3'
RII-2	5'-GAAAAGTTGGATAATTCAATG-3'
Asn519	5'-ATCCCACTGTTGGATATGSWCARRTA-3'
RFSHEXPfW2	5'-AAGCTTGAGATGATGAAGATGAAGAAGAT-3'
RFSHOZEXPrv	5'-AACTGGTTCAAATCAACCAAGATCTAGA-3'
RLHEXPfW	5'-CGATAATTCAATGATGTCGATA-3'
RLHEXPPrv2	5'-TTTCAGGTATAGGGTGCCATT-3'
RFSHICF2	5'-TCAGTCACCTGACGATCTGCAA-3'
RFSHICR2	5'-TCCTGCAGGTCCAGCAGAAACG-3'
RLHICF2	5'-CTTCTCAACCTCAATGAAATCTTC-3'
RLHICR2	5'-GGATATACTCAGATAACGCAGCTT-3'
28SFw1	5'-TGTGAACAGCAGTTGAACATGG-3'
28SRv1	5'-ATCTGAACCCGACTCCCTTT-3'

set efficiencies were $96.5 \pm 19.2\%$ for FSHR, $93.4 \pm 13.2\%$ for LHR, and $84.2 \pm 10.5\%$ for 28S rRNA. Correlation coefficients of the standard curves were 0.996 ± 0.001 , 0.997 ± 0.001 , and 0.996 ± 0.02 for FSHR, LHR, and 28S rRNA respectively. No amplification was observed from non-reverse-transcribed sample RNA (from testis at stages I–III, and VIII), indicating the absence of contaminant such as genomic DNA. Specificity of the PCR product was determined from the melting curve analysis (10 s holding followed by a 0.5°C increase, repeated 80 times, and starting at 55°C). The mean cycle threshold (C_T) was calculated for each sample using the iCycler software (Bio-Rad). The relative abundance of target cDNAs was first normalized with 28S rRNA using the well-established delta C_T method (Livak & Schmittgen 2001). No significant ($P > 0.05$) difference in the reference 28S rRNA gene expression was observed between stages of gonadal maturation either in males or in females. Data were analyzed using the nonparametric Mann–Whitney U test of the Statistica software (Statsoft, France).

Functional characterization of rainbow trout FSHR (rtFSHR) and LHR

The putative open reading frame, including the stop codon of each cDNA, was PCR amplified using RFSHEXPfw2/RF-SHOEXPRv and RLHEXPfw/RLHEXPv2 primer sets. The resulting PCR products were cloned into the pcDNA 3.1/V5-His-TOPO expression vector (Invitrogen) upstream from the polyadenylation site of the bovine growth hormone gene and downstream the cytomegalovirus (CMV) promoter. The inserts were entirely checked by DNA sequencing on both strands. To test the functionality of FSHR and LHR, we expressed each receptor in COS-7 cells that were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) newborn calf serum. In total, 500 000 cells, seeded onto six-well plates, were cotransfected with either pcDNA 3.1/V5-His-FSHR or pcDNA 3.1/V5-His-LHR (500 ng/well) together with $1\text{ }\mu\text{g/well}$ of the cAMP-responsive reporter construct pCRE-Luc (Stratagene) and 50 ng/well pCMV β -galactosidase (Clontech) using $3\text{ }\mu\text{l/well}$ of FuGENE6 reagent (Roche Applied Science). Note that DNA quantity was adjusted to $2\text{ }\mu\text{g}$ using the pGEM-T vector (Promega). Seven hours after transfection, cells were trypsinized and replated on 24-well plates overnight. Twenty-four hours after transfection, cells were stimulated with purified GTHs for six hours. Incubations were stopped by washing the cells twice with PBS $1\times$ and adding $100\text{ }\mu\text{l}$ $1\times$ cell lysis buffer (Promega Corporation). Cells were incubated for 15 min under agitation (100 g) at room temperature, and frozen at -20°C until luciferase activity was measured from $20\text{ }\mu\text{l}$ lysates using the luciferase assay kit (Promega). The β -galactosidase activity was determined according to the manufacturer's instructions (Promega) from $30\text{ }\mu\text{l}$ cell lysates. Each stimulation was performed in triplicate and each experiment repeated at least twice. The hormone concentrations inducing half-maximal stimulation (EC_{50}) were calculated using the GraphPad Prism 4

software package (GraphPad Software Inc., San Diego, CA, USA). Rainbow trout and chinook salmon (*Oncorhynchus tshawytscha*) GTHs were purified using metal ion affinity chromatography and dye–ligand chromatography (Govoroun *et al.* 1997). The homogeneity and specificity of the GTH preparations were checked by reverse-phase high pressure liquid chromatography (Govoroun *et al.* 1997) and RIA (Govoroun *et al.* 1998).

Sequence analysis

The amino acid alignments of the sequences with other known G-protein-coupled receptors were performed using CLUSTALW and BioEdit shareware. The signal peptide cleavage site was predicted at the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). The seven membrane-spanning regions were predicted at the server TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). Prediction of the Ser, Thr, and Tyr phosphorylation sites was carried out at <http://www.cbs.dtu.dk/services/NetPhos/>. The potential N-glycosylation sites were predicted at <http://www.cbs.dtu.dk/services/NetNGlyc/>.

Statistical analysis

Data were analyzed using the nonparametric Mann–Whitney U test of the Statistica software based on the ranking method.

Results

Cloning of transcripts encoding proteins related to the GTH receptors

To characterize the rainbow trout GTH receptors, the molecular cloning of the corresponding transcripts was undertaken. Two full-length cDNA were isolated from trout testis as described in the Materials and Methods section. Figure 1 shows the deduced amino acid sequence from the open reading frame of each cDNA. The encoded proteins share poor overall homology (42% identity/56% homology). A search for conserved functional domains and motifs showed that the putative proteins have a structure similar to that of the GPCR superfamily and, in particular, to the glycoprotein hormone receptors that belong to the δ -subfamily (Fredriksson *et al.* 2003). Amino acid sequence alignment and phylogenetic analyses with known GPCR showed strong overall homology with the vertebrate GTH receptors (Fig. 2). The distinct cDNA segregated into two different clades, corresponding to each of the GTH receptor types. Therefore, the cDNA were named according to the homologous GTH receptor type. The FSHR cDNA length is 2783 bp and the open reading frame encodes 658 aa corresponding to a 73.7 kDa translated protein. The LHR cDNA is 2756 bp and harbors a 728 aa open reading frame corresponding to an 80.75 kDa translated protein.

Figure 1 Amino acid sequences of rainbow trout FSHR (GenBank accession number AAQ04551) and LHR (GenBank accession number AAQ04550). The predicted signal peptides at the N-terminal are underlined and the putative cleavage sites are indicated with a broken arrow. Boxes and asterisks represent the potential N-glycosylation and phosphorylation sites respectively. The seven transmembrane helices are shown in grey.

The highest similarity for both trout GTH receptors (99%) is displayed by the Atlantic salmon (*Salmo salar*) and the amago salmon (*O. rhodurus*) GTH receptors. Homologies with other FSHR among teleost fish remain rather high ranging from 75 to 80%. The overall homology of trout FSHR with other vertebrate counterparts decreases to around 70% (human, reptiles, batrachians, birds). Interestingly, LHR appears to be better conserved among fish species (from 83 to 87%) with the exception of the tilapia, *Oreochromis niloticus* (71%), and the channel catfish, *Ictalurus punctatus* (69%). A similar homology with other vertebrates (mammals, birds) is observed which ranges from 69 to 73%.

A structural analysis of the putative rtFSHR and rtLHR amino acid sequences indicates the presence of three main functional regions: extracellular, transmembrane, and intracellular domains. A large extracellular domain that displays similar features to the leucine-rich glycoprotein receptors (LGR) is located at the N-terminal end. This extracellular domain includes a 23 (rtFSHR) to 27 (rtLHR) amino acid region that is highly hydrophobic and encompasses a putative signal peptide, as revealed using the sliding window/matrix scoring method and -1, -3 rule for signal peptide prediction. A cluster of nine repeated sequences is observed on each receptor (Fig. 3). These sequences are related to imperfect

leucine-rich repeats (LRRs) and most likely form a succession of β -strands and α -helices, organized into a horseshoe-shaped structure. These nine LRRs, conserved in sequence and position between rtFSHR and rtLHR, are similar in length and sequence to the typical LRR (Kajava 1998). Interestingly, the proximal cysteine-rich flanking region that normally links the N-terminal end of the protein to the LRR clusters in mammalian FSHR and LHR, is not found in the rtFSHR sequence. This structural change is observed in salmoniform and perciform FSHR, whereas in zebrafish and catfish, only two of the four cysteine residues liable to form a disulfide bond are present (Fig. 4). The distal cysteine-rich domain that precedes the transmembrane domain is present in both receptors, and is close to the C-terminal cysteine-containing flanking domain 3 (CF3) domain consensus described by Kajava (1998).

The extracellular domain displays five and three potential N-glycosylation sites in rtFSHR and rtLHR respectively (Fig. 1). Only the glycosylation motif (NGT) is conserved in the three glycoprotein hormone receptors out of all species studied so far.

As observed in other vertebrate GTH receptors, the transmembrane domain is the most conserved in terms of sequence and size and is composed of seven hydrophobic regions, forming a tertiary structure of short α -helices. Spacer regions that have been involved in extracellular and intracellular loops are also well conserved. Finally, the C-terminal end forms an intracellular domain. The intracellular domain of the rtLHR includes five putative tyrosine or serine/threonine kinase phosphorylation sites. The intracellular domain of the rtFSHR is shorter than that of the rtLHR (52 aa versus 72 aa) and this feature is also observed in other fish species. In addition to the shorter size, only two putative phosphorylation sites for serine/threonine kinases are found.

Pharmacological characterization of rainbow trout GTH receptors

The functionality of the trout putative GTH receptors was studied by transient transfection assays in COS-7 cells expressing either one of the receptors. Since it is well established that GTHs

induce intracellular cAMP production upon binding to their receptors, an indirect quantification of intracellular cAMP production was carried out using a cAMP-responsive luciferase reporter gene. The high concentration (800 ng/ml) of purified fish GTHs used in this study did not modulate reporter gene expression in the absence of GTH receptors (Fig. 5A). Transfection of the cells with increasing amounts of the expressing vectors (from 0 to 500 ng/well) showed that, in the absence of a ligand, both trout GTH receptors had no constitutive activity (Fig. 5B and C).

Transactivation data, obtained upon incubation in the presence of GTHs, are reported in Fig. 6 and maximal induction values are indicated in Table 2.

Regarding rtFSHR, a dose-dependent response curve was observed using rtFSH at a concentration of 25–1000 ng/ml (Fig. 6). A significant ($P < 0.05$) maximal 2.5-fold induction (Table 2) of the reporter gene expression was seen at 100 ng/ml. No reporter gene induction was observed in presence of rtLH. Similar results were obtained using chinook GTHs (cFSH and cLH). cFSH was able to activate rtFSHR to a similar level (2.4-fold induction) but at a higher dose compared with rtFSH (400 vs 100 ng/ml). cFSH potency appeared to be much lower with a calculated effective half-maximum concentration (EC_{50}) equal to 200 ng/ml versus 38 ng/ml for rtFSH. As observed for rtLH, cLH was not able to induce reporter gene expression regardless of the concentrations used (Fig. 6).

Regarding rtLHR, rtLH induced luciferase reporter gene expression in a dose-dependent manner from 25 to 1000 ng/ml (Fig. 6). The rtLHR was highly responsive, showing a significant ($P < 0.05$) 8.9-fold induction at 1000 ng/ml, and an EC_{50} of 117 ng/ml. Interestingly, high doses of rtFSH (1000–1600 ng/ml, data not shown) were also able to induce a significant ($P < 0.05$) and reproducible twofold induction of the reporter gene expression. However, the biopotency of rtFSH in activating rtLHR was 16-fold lower compared with that calculated for rtFSHR (EC_{50} = 598 vs 38 ng/ml for rtLHR and rtFSHR respectively). The heterologous chinook GTHs were also tested. cLH induction resulted in a similar dose-dependent responsiveness to that obtained in the presence of rtLH. The biopotency of cLH was similar to that of rtLH with an

Figure 2 Phylogenetic analysis of rainbow trout gonadotropin receptors. Amino acid sequences of known or predicted glycoprotein hormone receptors were first aligned using CLUSTALW, and the resulting alignment was analyzed using the MEGA software. The analysis was carried out on full-length protein sequences using two different algorithms (minimum evolution and neighbour-joining methods). Similar trees were obtained but only the analysis performed by the neighbour-joining method is represented. The numbers beside the branches indicate bootstrap values from 100 replicates. The scale bar corresponds to estimated evolutionary distance units (MYA). The outgroup used to build the tree is the sea anemone leucine-rich repeat containing G-protein-coupled receptor (Z28332). TSHR sequence accession numbers are AY533543 (channel catfish), AY129556 (African catfish), AF239761 (striped bass), AB030954 (TSHRA, amago salmon), AB030955 (TSHRB, amago salmon), fugu (unpublished results), tetraodon (unpublished results), NM_012888 (rat), BC092523 (mouse), NM_001009410 (sheep), NM_174206 (bovine), AF218264 (cat), NM_214297 (pig), AY169400 (monkey), and AY429111 (human). LHR sequences are AF324540 (African catfish), AY424302 (zebrafish), fugu (unpublished results), AY642114 (sea bass), AF439404 (rainbow trout), AB030005 (amago salmon), AB041763 (tilapia), AJ579790 (Atlantic salmon), AY587261 (sea bream), NM_204936 (chicken), NM_012978 (Rat), U20504 (bovine), and NM_000233 (human). FSHR sequences are AY587262 (sea bream), AJ567667 (Atlantic salmon), AF285182 (channel catfish), AB030012 (amago salmon), AF439405 (rainbow trout), AY278107 (zebrafish), AY642113 (sea bass), AB041762 (tilapia), AJ012647 (African catfish), AY189696 (snake), AB005587 (newt), AJ292553 (lizard), NM_205079 (chicken), AY099289 (duck), AY082002 (tammara wallaby), AY509907 (hamster), AY521181 (cat), U73659 (donkey), NM_001009289 (sheep), NM_214386 (pig), XM_001114171 (monkey), and M65085 (human). Note that rainbow trout cDNA encoding glycoprotein hormone receptors segregate into two distinct clades, corresponding to the FSHR and LHR genes.

LHR	RGPRLVLKHLTMST-IASHTFDGLR	56- 79
FSHR	T-TDLEFKQTHIRV-FPREAFTNLQ	54 - 76
LHR	RVQHIEIGQSVALETIETLAFNNLL	80 - 104
FSHR	QLTAIVLTENGMLESIGAFANLP	77 - 101
LHR	NLNEIFIKNIRSLVHIARRTFNNLP	105 - 129
FSHR	RLTEITITKSKHLVIIHHQAFIGLP	102 - 126
LHR	KLRYLSIS-NTGITVFPDMTSIHSLEPWN	130 - 157
FSHR	KLSHLTIC-NTGLRVLPNFSRIHSAA	127 - 151
LHR	QNFDLDICDNLVLLSIPVNAFVGMTT	158 - 183
FSHR	LTFLDLQDNVHIVIIPS-AFLGLTTN	152 - 178
LHR	EYTAMNLFNNG-IREIQDYAFNGT	184 - 206
FSHR	TIDELRLTKNG-ISEVESHAFNGT	179 - 201
LHR	KINKLVLKNNRNLRVIHREAFKGAV	207 - 231
FSHR	KIKKLFMLGNLQLSHMHNNSEFKGAE	202 - 226
LHR	GPRILDVSSA-IETLP--SHGLN	232 - 252
FSH	GPGFLDISRTA-LSSLPE-SVLGE	227 - 248
LHR	SVVELVARTAYGLKRLPFRDLG	253 - 275
FSHR	-VEHLSAVSVFSLRALPPLSLFTK	249 - 271
Cons LRR	xLxxLxLxxNx-LxxLPxxxFxx

Figure 3 The nine repeat sequences found in the extracellular domains of rainbow trout FSHR and LHR are related to imperfect leucine-rich repeats (LRRs). Spaces have been added to improve the alignment. The consensus sequence of LRR (Kajava *et al.* 1995) is indicated below the alignments. A schematic of the extracellular domains is shown at the bottom which indicates the position of different structural motifs: signal peptide, the LHR N-terminal cysteine-rich flanking region containing four cysteine residues (c), the cluster of imperfect LRR numbered 1–9, and the C-terminal cysteine-rich flanking region. The amino acids delineating the ectodomain are indicated. Note the absence of the N-terminal cysteine-rich flanking region on rtFSHR.

Receptor type	N-terminal sequences (including the signal peptide) alignment									
FSHR trout	----	MMKMKK	IMKMLLCMLG	CVCVSQ----	-----	AEVA	MVNSGTTF	TY	LCMGN	TITAI VLTE 50
FSHR salmo salar	----	MMKMKK	IMKMLLCVLG	CVCVSQ----	-----	AEVA	MVNSGTTF	TY	LCMGN	TITAI VLTE 50
FSHR amago	----	MMKMKK	IMKMLLCVLG	CVSMVSQ----	-----	AEVA	MVNSGTTF	TY	LCMGN	TITAI VLTE 50
FSHR seabass	----	MMVMIM	LIMLMILMIK	TATASVPGPE	MDVKPGVETS	LAKRTLSFCY	QLKFG-VRKL	TIWE	59	
FSHR tilapia	----	MMLVMT	LMMLLIVTIK	MAAASAHGSE	MDIRPGFHPS	LAKQTSCLSY	QVMFG-VMEL	TISE	59	
FSHR zebra	MVLSMMLCFI	LGCS	INTANT	TLAASQ----	-----	F	CAFNGSTRSF	ICLGN	KVKRI VVSE 51	
FSHR c catfish	----	MMCFI	LSWLMMHAGN	MCLGSY----	-----	A	CLANGTTRSF	ICLGS	KVKRI LVSE 46	
FSHR af catfish	----	MLRYI	LSWLVMHTGN	MFLGSY----	-----	A	CLASGTTRSF	ICLGS	KVKRI MVSE 46	
FSHR rat	----	MALLL	VSLLAFLGTG	SGCHHW----	-----	-LCHC	SNRVF	LCQDS	KVEKI EISQ 44	
FSHR human	----	MALLL	VSLLAFLSLG	SGCHHR----	-----	-LCHC	SNRVF	LCQES	KVEKI EISQ 44	
FSHR bovin	----	MALLL	VALLAFLSLG	SGCHHR----	-----	-LCHC	SNGVF	LCQES	KVEKI EISQ 44	
FSHR chicken	----	MSLGL	TCLLILLASC	SGCQHH----	-----	-TCLC	EGRIF	ICQEI	KVEKI EISQ 44	
FSHR duck	----	MFLVF	TCSLILLASC	SSCQHH----	-----	-TCHC	AGRIF	ICQES	KVEKI EISQ 44	
LHR trout	----	MMSISL	LFLFYPSVLL	FFFGGCGYTS	SFVCPGICRC	SAN-TIR	CNN	ITEK	49	
LHR salmo salar	----	MMSISL	LFLFYPSVLL	FFFGGCRYAS	SFVCPGICRC	SSN-TIR	CNN	ITEK	49	
LHR amago	----	MSISL	LFLFYPSVLL	FFFGGCRYAS	SFVCPGICRC	SAN-TIR	CNN	FTEK	48	
LHR seabass	----	MWTSL	PALLFLSVLG	FY--GCKCAP	GFGCPRICRC	FSN-TIR	CNN	VTQG	46	
LHR tilapia	----	MALRE	VWLLFALSGV	LN--ARSCC	AYTCAI	CRC	TAD-SFQ	CSK ETQL 45		
LHR zebra	----	MWSAL	LLVFLLLT--	----SFCCGV	CFECPEI	CRC	SQK-SIT	CNS ATGS 43		
LHR c catfish	----	MVSRC	SVAALLLAVV	MR--LSRGG	AFTCPPICSC	TAD-TLS	CTA QTER 45			
LHR af catfish	----	MRTSL	FILIVMMC--	----QCDC-L	RFVCP	EVCR	SIK-TIS	CNS ATEA 41		
LHR rat	MGRRPALRQ	LLVL	AVLLLK	PSQLQSRELS	GSRCP	EP	CD	APD	GALRCPG PRAG 54	
LHR human	MKQRF	SALQ-	-LLKLL	LLQ P--PLPRALR	EALC	PE	FCNC	VPD	GALRCPG PTAG 50	
LHR bovin	MGRPSLALR-	-LLL	ALLLLP	PPAPLLWALR	PAPC	PE	FCSC	PPD	GALRCPG PQAG 52	
LHR chicken	-----	-MLP	ALLPLL	LPALLPG-AG	GGRCP	QR	CAC	TQP-ALR	CPT PPPG 41	

Figure 4 Alignment of the rtFSHR and rtLHR N-terminal cysteine-rich flanking regions with those of other piscine and mammalian species. Conserved cysteine residues are shown bold in grey boxes. c catfish, channel catfish; af catfish, African catfish.

EC₅₀ equal to 98 vs 117 ng/ml for rtLH (Table 2), with an 11-fold maximal induction obtained at 400 ng/ml. Unexpectedly, contrary to rtFSH, cFSH had no significant effect on rtLHR transactivation at the tested doses.

In summary, functional data indicate that, in our assay system, rtLHR and rtFSHR showed a marked functional specificity to their cognate ligand.

Expression pattern of the trout GTH receptor genes in the gonads

To provide new insight into trout GTH receptor function, the expression pattern of their genes was studied over the annual reproductive cycle in female and male trout using quantitative real-time PCR. In females, rtFSHR and rtLHR transcripts were present in all stages studied (Fig. 7); however, different expression patterns were found for the two messengers prior to spawning (Fig. 7B). The relative abundance of rtLHR transcript significantly increased during the ovulation period (fivefold, $P < 0.05$). Unexpectedly, the relative abundance of rtFSHR peaked significantly ($P < 0.05$) earlier, just before spawning at final oocyte maturation and ovulation (sixfold compared with the end of vitellogenesis).

We previously showed an inverse evolution of GTH plasma levels after ovulation, dependent on whether the eggs were retained in the abdominal cavity (Breton *et al.* 1998). FSH secretion significantly increased after ovulation in fish without eggs, whereas in fish that retained eggs, FSH secretion remained constant. The LH profile was exactly the opposite, being significantly higher in fish with eggs compared with those that were stripped. In order to determine whether a modification in the plasma hormonal balance, induced after ovulation by egg removal, correlates with changes in GTH receptor gene expression, we examined the relative abundance of ovarian rtFSHR and rtLHR transcripts in ovulated females with or without eggs retained in the abdominal cavity after ovulation (Fig. 7C). The relative abundance of rtFSHR transcripts significantly decreased after ovulation (fivefold decrease, $P < 0.05$). This decrease seemed to be slightly delayed in females that retained eggs in the abdominal cavity, but a significant difference between the two groups (twofold, $P < 0.05$) was only detected 10 days after ovulation. On the contrary, rtLHR transcript did not change significantly for up to 15 days after ovulation, and no significant difference was observed in females with or without eggs.

Figure 5 Analysis of the basal activity of rainbow trout gonadotropin receptors. COS-7 cells were cotransfected with increasing amounts (from 0 to 500 ng/well) of native (A) or trout gonadotropin receptor (B and C) expression vectors, together with the cAMP-responsive reporter construct pCRE-Luc. Cells were incubated in the absence of gonadotropins or were stimulated for 6 h in the presence of 800 ng/ml FSH (+rtFSH) or LH (+cLH) as a positive control. cAMP production was indirectly quantified by measuring the luciferase activity from the reporter vector. Each data point represents the mean $\pm$ s.d. of triplicates of a representative experiment.

In males, rtFSHR and rtLHR transcripts were also present in all the studied stages and their expression followed a similar pattern (Fig. 8). In immature fish and during the gonial proliferation period (stages I and II), the relative abundance of both transcripts did not change significantly. Starting from meiosis onset to full spermiogenesis, there was a progressive and significant ($P < 0.05$) decrease in rtFSHR and rtLHR

transcripts (8- and 12-fold decrease respectively). However, normalization of the data corrected for the reference gene, the gonadosomatic index and RNA extraction yields indicate no significant change in the rtFSHR and rtLHR messenger levels during spermatogenesis. Interestingly, independent of the normalization procedure used, both transcripts reached their maximum expression at spermiogenesis (four- to fivefold increase for rtFSHR and rtLHR respectively compared with the immature gonadal stage I). In post-spawning males (stage IX), rtFSHR levels returned to nearly immature levels, whereas rtLHR messengers continued to be expressed at a level five times greater than that in immature fish. Normalizing the data for the gonadosomatic index and RNA extraction yields showed that the accumulation of the GTH receptor transcripts remained high in post-spawning/regressing male compared with immature fish.

Discussion

Structure of trout GTH receptors

In the present study, we identified two distinct cDNA encoding rainbow trout GTH receptors based on the analyses of their amino acid primary sequences and on *in vitro* functional studies with homologous and heterologous purified GTHs. The presence of high-affinity receptors for LH was previously demonstrated in male and female trout from *in vitro* binding assays to membrane preparations or purified GTH receptors (Quesnel & Breton 1993). Since no binding study has been carried out with purified trout FSH, clear evidence for a second distinct GTH receptor type in trout relied mainly on *in vitro* biological studies where purified FSH was shown to act on specific gonadal functions. Loir (1994, 1999) showed that purified rtFSH, but not rtLH, was a potent mediator of trout spermatogonia proliferative activity *in vitro*.

The presence of two genes encoding distinct GTH receptors in trout is in agreement with previous reports on other fish species, including amago salmon (Oba *et al.* 1999a,b), Atlantic salmon (Maugars & Schmitz 2006), African catfish (Bogerd *et al.* 2001, Vischer & Bogerd 2003), channel catfish (Kumar *et al.* 2001a,b), and zebrafish (Laan *et al.* 2002, Kwok *et al.* 2005). Analysis of the overall architecture indicates that the two trout receptors should be considered as new members of the GPCR superfamily and, in particular, to the glycoprotein hormone receptor (GpR) subfamily. Members of the GpR subfamily are characterized by a large N-terminal extracellular domain (ectodomain), a conserved seven α -helix transmembrane region, and a short C-terminal intracellular tail (Fredriksson *et al.* 2003). Each trout GTH receptor is highly similar to that of the few other fish counterparts identified so far, suggesting that two distinct GTH receptors are well conserved among euteleosts. Moreover, with the exception of the pCRR structural domain located at the N-terminal end of the mammalian FSHR, fish GTH receptors share similar structural motifs with their

Figure 6 Transient expression and hormone responsiveness of rainbow trout FSHR and LHR. COS-7 cells were cotransfected with trout gonadotropin receptor expression vectors together with the cAMP-responsive reporter construct pCRE-Luc. Cells were incubated for 6 h with increasing doses of either rainbow trout (A and B) or chinook salmon gonadotropins (C and D). Hormone-induced cAMP production was indirectly quantified by measuring the luciferase activity from the reporter vector. Each data point represents the mean $\pm$ s.d. of triplicates of a representative experiment.

reptilian, avian, and mammalian counterparts, indicating that they have been well conserved in all vertebrates during evolution. This assumption is also supported by the molecular phylogenetic analysis of the known vertebrate glycoprotein receptors, indicating that the two receptor types have emerged from a common ancestral gene.

In vitro hormonal specificity of the trout GTH receptors

To characterize their hormonal specificity at a functional level, trout GTH receptors were overexpressed in COS-7 cells together with a cAMP-responsive luciferase reporter gene and stimulated with an increasing amount of homologous or

heterologous purified GTHs. As expected, the endogenous ligands were more potent in stimulating their cognate receptor, but the calculated EC_{50} required to transactivate rtFSHR (rtFSH: 38 ng/ml) and rtLHR (rtLH: 117 ng/ml) appeared to be higher than trout plasma GTH levels, which range from 2 ng/ml in juvenile to 15 ng/ml in sexually mature animals (Gomez *et al.* 1999). It is not known whether the apparent high EC_{50} , required to activate fish GTH receptors *in vitro*, is due to reduced biological hormone activities resulting from purification/conservation procedures.

Although rtFSH preferentially activates its own receptor, the rtFSHR maximal response to rtFSH (2.5-fold) was much lower than that of rtLH on rtLHR (about 9-fold). However, such a

Table 2 Effective half-maximum concentration (EC_{50}) and maximum induction calculated for each rainbow trout gonadotropin receptor. The maximum induction values are relative to the basal expression of the reporter gene measured in the absence of hormone. Data are the mean $\pm$ s.d. of three replicates of a representative experiment

	FSHR		LHR	
	EC_{50} ng/ml	Maximal induction	EC_{50} ng/ml	Maximal induction
Hormones				
rtFSH	38	$2.5 \pm 0.1^*$	598	$2.9 \pm 0.1^*$
cFSH	200	$2.4 \pm 0.3^*$	na	1.0 ± 0.2
rtLH	na	1.2 ± 0.2	117	$8.9 \pm 0.9^*$
cLH	na	1.8 ± 0.6	98	$11.0 \pm 2.1^*$

*Significant induction $P < 0.05$; na, not applicable.

Figure 7 Expression profiles of female rainbow trout FSHR and LHR transcripts measured by quantitative real-time PCR during the first annual reproductive cycle from early to mid-vitellogenesis (A) and from the end of vitellogenesis to ovulation (B). Each gonadotropin receptor transcript was studied after the ovulation period (C). Expression levels were normalized to 28S rRNA. The black bars represent females stripped on the day of ovulation. Hatched bars represent females with eggs retained in the abdominal cavity for 5, 10, or 15 days after ovulation. * Asterisks represent significant difference ($P < 0.05$) compared with D0. The closed diamond symbol indicates significant differences ($P < 0.05$) between females with or without eggs retained in the abdominal cavity. Each data point represents the mean $\pm$ S.E.M. of different individual fish. Bars with different letters are significantly different ($P < 0.05$). Number of individual fish is indicated between brackets ($n = 5-11$). BV, beginning vitellogenesis; MV, mid-vitellogenesis; EV, end vitellogenesis; Mat, maturation; Ov, ovulated females between 0 and 3 days.

weak FSH-induced response was previously reported in amago salmon using purified (Oba *et al.* 1999b) or recombinant (Ko *et al.* 2007) heterologous GTH regardless of the direct and indirect intracellular cAMP quantification systems used. Similar

observations were also reported in catfish (Kumar *et al.* 2001b) and zebrafish (Kwok *et al.* 2005, So *et al.* 2005).

In our assays, rtLHR was activated not only by rtLH ($EC_{50} = 117$ ng/ml) but also by rtFSH, albeit at doses at least

Figure 8 Expression profiles of male rainbow trout FSHR (C and E) and LHR (B and F) transcripts measured by quantitative real-time PCR during the first annual reproductive cycle: from immature (stage I) to post-spermiation (stage IX). The gonadosomatic index (A) and calculated total RNA content per testis (B) are presented. Expression data were normalized to 28S rRNA (C and D) or the gonadal RNA content (E and F) as described previously (Kusakabe *et al.* 2006). Each data point represents the mean $\pm$ S.E.M. ($n=3-4$). The number of individual fish is given in parentheses. Bars with different letters are significantly different ($P<0.05$).

five times higher ($EC_{50}=598$ ng/ml). In addition, cells expressing rtLHR showed a high maximum response in the presence of rtLH (8.9-fold) that was significantly reduced in the presence of rtFSH (2.9-fold), independent of the dose used (up to 800 ng/ml). However, the activation of the rtLHR by homologous FSH was not observed upon stimulation by the heterologous chinook FSH. Contamination of the purified rtFSH fractions by rtLH may account for such an activation, although no trace of rtLH was detected

by radio immunoassay in the purified rtFSH preparation (Govoroun *et al.* 1998). Other hypotheses include a weaker affinity of the heterologous hormone for the trout GTH receptors. This hypothesis is supported by the fact that the chinook FSH preparation used in the study specifically activated rtFSHR, but higher doses were required compared with the homologous rtFSH preparation. Unfortunately, we could not test very high concentrations because of limited availability of purified chinook GTHs.

Interestingly, the functional specificity of the trout GTH receptors is consistent with the reports in amago salmon, showing that, *in vitro*, a unique high concentration (5 µg/ml) of purified heterologous LH or FSH preferentially activates their cognate receptors (Oba *et al.* 1999a,b). These observations were recently confirmed using recombinant Manchurian trout GTHs (Ko *et al.* 2007). The prominent selective activation of the salmonid GTH receptors is rather unexpected since previous GTH-binding studies on membrane preparations from pacific salmon gonads demonstrated that FSHR bound both hormones, whereas LHR bound LH with a rather high selectivity (Yan *et al.* 1992). The difference observed between the results of the binding and activation studies suggests that LH binding to FSHR would result in a ligand/receptor complex unable to activate the adenylate cyclase in the COS cellular context. In addition, we cannot rule out the hypothesis that rtLH/FSHR complexes may activate other unknown signaling pathways.

In zebrafish, the functional specificity seems less prominent since recombinant zFfSH specifically stimulated its cognate receptor, whereas recombinant zfLH efficiently activated both GTH receptors (So *et al.* 2005). Similarly, in African catfish, recombinant cfFSH specifically stimulated cfFSHR whereas recombinant and purified cfLH were efficient in activating FSHR, although with a potency 10- to 20-fold lower (Vischer *et al.* 2003). Our study, together with other studies, indicates that the *in vitro* selective activation of the fish GTH receptors may differ depending on the fish species and/or order. In trout and amago salmon, FSHR and LHR would be specifically induced by their respective ligands, whereas in African catfish (Siluriforms) and zebrafish (Cypriniforms), which are two phylogenetically closely related species, FSHR would be efficiently activated by both hormones. In Siluriforms and Cypriniforms, but not in Salmoniforms, the N-terminal end of the FSHR exhibits two of four conserved cysteine residues present on a classical pCRR. These residues have been involved in disulfide bond formation and are part of a structural determinant required for efficient LH binding in mammals (Zhang *et al.* 1996). Whether these two conserved cysteine residues are involved in catfish and zebrafish FSHR folding and LH signal transduction remains to be investigated.

Expression of the GTH receptor genes in the gonad

In female trout, rtFSHR gene expression significantly increased at the maturation stage prior to ovulation. Such a FSHR gene expression pattern was also described in the tilapia (Oba *et al.* 2001) and zebrafish (Kwok *et al.* 2005). The physiological significance of high FSH levels and FSHR gene expression during oocyte maturation and spawning in salmonids remains puzzling. However, in mammals (Hoak & Schwartz 1980) and fish (Prat *et al.* 1996, Tyler *et al.* 1997), it has been proposed that FSH plays a role in follicle recruitment for the next reproductive cycle. Meanwhile, recombinant FSH injected into female rats induces not only follicle growth but also ovulation (van Cappellen *et al.* 1995).

In trout, Bobe *et al.* (2003) found a positive correlation between the relative abundance of rtFSHR transcript and high follicular maturational competence. The rtLHR gene expression pattern in the ovary showed a progressive increase in LHR transcript abundance from the end of vitellogenesis to ovulation. Such a pattern is similar to that described in zebrafish (Kwok *et al.* 2005). The higher expression of GTH receptors in maturing and ovulated females is consistent with studies showing higher binding sites (Breton & Sambroni 1989) and high plasma GTH levels (Breton *et al.* 1998) in sexually mature females. Altogether, these results agree with the known functions of LH on ovarian steroidogenic activity, oocyte maturation, and spawning (Patino & Sullivan 2002).

In male trout, FSHR mRNA was detected in early gonadal maturation stages at Sertoli cell proliferation and differentiation (stages I and II). The presence of rtFSHR at the beginning of the cycle reinforces the idea that FSH could mediate Sertoli cell proliferation, as proposed previously in catfish and tilapia (Schulz *et al.* 2005). In the present study, the relative abundance of rtFSHR mRNA appeared to decrease progressively as the proportion of germ cells increased in the gonad (stages III and VI). A similar pattern was observed for the LHR transcript. Are these apparent variations reflecting a down-regulation of the GTH receptor genes? Gonadal FSHR mRNA expression was found to decrease after exogenous androgen treatment in immature catfish (Schulz *et al.* 2003). We do not exclude that a similar hormonal regulation may occur in trout, since the decrease in relative abundance of rtFSHR transcript also coincides with a slow increase in plasma testosterone levels described previously in our trout strain between stages I and VI (Gomez 1998). However, if we assume that the GTH receptors are mainly expressed in somatic cells, the decrease in the relative abundance of the receptor transcripts would most likely be due to a 'dilution' effect. In fact, when the receptor mRNA abundance is expressed as total amount per testis, no significant decrease in rtLHR or rtFSHR abundance could be detected between stages III and VI of gonadal development. In a recent study, in which the transcript content was expressed per gonad, the amount of FSHR messengers was even found to increase coincidentally with gonadal growth and with the increase in germ cell differentiation (meiosis and spermiogenesis; Kusakabe *et al.* 2006). In that study, LHR gene expression also progressively increased as spermatogenesis progressed and was correlated with plasma LH levels. Further studies will be required to determine whether changes in the relative abundance of GTH receptor mRNA only result from changes in the proportion of expressing cells or whether they occur as a result of specific regulations in GTH target cells. Unfortunately, our attempt to locate and quantify trout GTH receptor transcripts using radioactive riboprobes on histological sections failed, most likely because of the low expression levels (Ricordel *et al.*, personal communication).

The expression pattern of trout GTH receptor genes shows a large increase in both receptor transcripts in stage VIII, regardless of the normalization procedure used. This increase

is consistent with studies showing higher LH-binding sites, higher sensitivity to LH in terms of steroid production output (Le Gac & Loir 1988, Planas & Swanson 1995), and high plasma GTH levels (Gomez *et al.* 1999) in prespawning or spawning males. The dramatic increase of rtLHR in stage VIII is also consistent with the fact that, using *in vitro* ligand autoradiography, LH binding could only be detected in spermiating salmon testis (Miwa *et al.* 1994).

To conclude, the present study describes the presence of two distinct GTH receptors in rainbow trout showing similarities with those of higher vertebrates, but also differences in terms of their structural determinants (FSHR). The gonadal expression pattern of the GTH receptor genes suggests that FSHR may play an important role in regulating gonadal functions in spawning trout, in addition to the LHR pathway. *In vitro* functional studies demonstrate that rtLH and rtFSH preferentially activate their cognate receptor.

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