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Characterization of gonadotropin receptors Fshr and Lhr in Japanese medaka, *Oryzias latipes*

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ABSTRACT

Reproduction in vertebrates is controlled by the brain-pituitary-gonad axis, where the two gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play vital parts by activating their cognate receptors in the gonads. The main purpose of this work was to study intra- and interspecies ligand promiscuity of teleost gonadotropin receptors, since teleost receptor specificity is unclear, in contrast to mammalian receptors. Receptor activation was investigated by transfecting COS-7 cells with either Fsh receptor (mdFshr, tiFshr) or Lh receptor (mdLhr, tiLhr), and tested for activation by recombinant homologous and heterologous ligands (mdFshβα, mdLhβα, tiFshβα, tiLhβα) from two representative fish orders, Japanese medaka (*Oryzias latipes*, Beloniformes) and Nile tilapia (*Oreochromis niloticus*, Cichliformes). Results showed that each gonadotropin preferentially activates its own cognate receptor. Cross-reactivity was detected to some extent as mdFshβα was able to activate the mdLhr, and mdLhβα the mdFshr. Medaka pituitary extract (MPE) stimulated CRE-LUC activity in COS-7 cells expressing *mdlhr*, but could not stimulate cells expressing *mdfshr*. Recombinant tiLhβα, tiFshβα and tilapia pituitary extract (TPE) could activate the mdLhr, suggesting cross-species reactivity for mdLhr. Cross-species reactivity was also detected for mdFshr due to activation by tiFshβα, tiLhβα, and TPE, as well as for tiFshr and tiLhr due to stimulation by mdFshβα, mdLhβα, and MPE. Tissue distribution analysis of gene expression revealed that medaka receptors, *fshr* and *lhr*, are highly expressed in both ovary and testis. High expression levels were found for *lhr* also in brain, while *fshr* was expressed at low levels. Both *fshr* and *lhr* mRNA levels increased significantly during testis development. Amino acid sequence alignment and three-dimensional modelling of ligands and receptors highlighted conserved beta sheet domains of both Fsh and Lh between Japanese medaka and Nile tilapia. It also showed a higher structural homology and similarity of transmembrane regions of Lhr between both species, in contrast to Fshr, possibly related to the substitution of the conserved cysteine residue in the transmembrane domain 6 in medaka Fshr with glycine. Taken together, this is the first characterization of medaka Fshr and Lhr using homologous ligands, enabling to better understand teleost hormone-receptor interactions and specificities. The data suggest partial ligand promiscuity and cross-species reactivity between gonadotropins and their receptors in medaka and tilapia.

1. Introduction

In teleosts as in other vertebrates, reproduction is controlled by the brain-pituitary-gonad (BPG) axis (Weltzien et al., 2004), wherein the pituitary-derived gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play essential regulatory roles. Fsh and Lh are heterodimeric glycoproteins, comprising a common α-subunit, and a β-subunit that confers biological specificity (Pierce and Parsons, 1981). In general, Fsh is important for early stages of gametogenesis, whereas Lh is mostly involved in processes leading to final gametogenesis, oocyte maturation and ovulation in females and spermiogenesis and spermiation in males (Yaron et al., 2003). Fsh and Lh act via activation of their cognate receptors expressed on gonadal cells. The specific membrane-bound gonadotropin receptors in fish were first

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reported in the early 1970s from binding studies using mammalian gonadotropins (human chorionic gonadotropin, hCG) and partly purified hypophysial glycoprotein hormones (Breton et al., 1973). A two-receptor model for teleost gonadotropins was suggested by the mid-1990s (Miwa et al., 1994), later supported by the molecular cloning of two distinct receptor cDNAs in amago salmon (*Oncorhynchus rhodurus*; Oba et al., 1999a,b), and several other species: Atlantic salmon (*Salmo salar*; Maugars and Schmitz, 2006), African catfish (*Clarias gariepinus*; Bogerd et al., 2001; Vischer and Bogerd, 2003), zebrafish (*Danio rerio*; Kwok et al., 2005), European seabass (*Dicentrarchus labrax*; Rocha et al., 2007a), Japanese eel (*Anguilla japonica*; Jeng et al., 2007), and rainbow trout (*Oncorhynchus mykiss*; Sambroni et al., 2007). Recently, a study based on genome analysis demonstrated the presence of a single Fsh receptor (*fshr*) gene and duplicated Lh receptor (*lhr*) genes in several teleost species resulting from a local duplication event early in the actinopterygian lineage (Maugars and Dufour, 2015). In the genome of Nile tilapia *(Oreochromis niloticus*), a single *fshr* and duplicated *lhr* genes, *lhr1* and *lhr2*, were found. In Japanese medaka (*Oryzias latipes*), another ovalentaria species, only single genes for *fshr* and *lhr* were conserved, the latter from the type1 (*lhr1*), whereas the second type (*lhr2*) was lost recently, after the emergence of the ovalentaria lineage (Maugars and Dufour, 2015).

Fshr and Lhr are both G-protein-coupled receptors (GPCRs), belonging to the family of rhodopsin-like receptors (family A) (Gether, 2000). They comprise, together with the thyroid-stimulating hormone receptors (Tshrs), the subfamily of glycoprotein hormone receptors, characterized by a large N-terminal extracellular domain (ECD), a seven transmembrane domain and a short intracellular C-terminal domain (Ji et al., 1998; Vassart et al., 2004). The extracellular domain contains at the N-terminal several leucine-rich repeats (LRRs) that are involved in the high binding specificity, and ends with a hinge region connecting to the transmembrane domain. Characteristic of the perciform Fshr is an extra LRR that potentially affects their ligand binding mode (Maugars and Schmitz, 2006; Rocha et al., 2007b). The receptor activation results from specific hormone binding that promotes the rearrangement of the transmembrane domain from an inactive to an active conformational state, which is coupled to the activation mainly of the Gs/cAMP/PKA pathway (Ji et al., 1998; Vassart et al., 2004).

In mammalian species, FSHR and LHR bind their respective ligands specifically and with little cross-activation (0.01–0.1%), leading to functional specificity in the presence of physiologically relevant hormone concentrations (reviewed in Levavi-Sivan et al., 2010). In contrast to mammals, various studies in teleosts indicate that gonadotropin receptors are less specific. This was shown first for female coho salmon (*Oncorhynchus kisutch*) demonstrating that Fshr, present on both theca and granulosa cells, did not distinguish between Fsh and Lh binding, whereas Lhr, localized on granulosa cells, bound only Lh (Miwa et al., 1994; Yan et al., 1992). Functional studies revealed that chum salmon (*Oncorhynchus keta*) Fsh preferentially activates amago salmon Fshr but also revealed some degree of interaction with amago salmon Lhr, whereas chum salmon Lh activates only amago salmon Lhr (Oba et al., 1999a,b, 2001). Later, there have been many functional studies on cloned teleost gonadotropin receptors using transfected mammalian cell lines (e.g. African catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003; Vischer et al., 2004), channel catfish (*Ictalurus punctatus*) (Kumar et al., 2001a,b), zebrafish (So et al., 2005), rainbow trout (Sambroni et al., 2007), European seabass (Molés et al., 2011), Japanese eel (Kazeto et al., 2008), Atlantic salmon (Andersson et al., 2009), and Nile tilapia (Aizen et al., 2012b)). From these studies, a more general pattern emerged. Teleost Lhrs tend to be specific for Lh, similar to their mammalian counterparts. Teleost Fshrs, however, possess a broader but still limited functional selectivity for both gonadotropins that may depend on the fish species or taxa (Aizen et al., 2012b). Nevertheless, more work is necessary to assess the physiological significance of the gonadotropin receptor cross reactivity in fish, and to explain the evolutionary processes behind that gave rise to Lhr, which is selective for Lh, and Fshr that can be activated both by Lh and Fsh for some species (Levavi-Sivan et al., 2010).

Previous studies on medaka gonadotropin receptors (Ogiwara et al., 2013) and receptors of other teleost species (Levavi-Sivan et al., 2010) suggest that Fshr is absent in the large preovulatory follicles that are destined to ovulate, supporting the hypothesis that Fsh/Fshr determines early phases of gametogenesis including vitellogenesis. In contrast, Lh/Lhr is suggested to stimulate the final stages, such as final oocyte maturation and ovulation (Ogiwara et al., 2013). However, little is known about the expression of Fshr and Lhr during puberty in male medaka. A previous study on medaka recombinant Lhr and Fshr using mammalian heterologous gonadotropins suggest that they appear to be specific for their cognate gonadotropins, compared to those from other teleost species (Ogiwara et al., 2013).

Most studies have performed ligand-receptor interaction studies in mammals, therefore teleost receptor specificity is not fully understood (Levavi-Sivan et al., 2010). Recent advances in the availability of piscine recombinant gonadotropins, in addition to the cloning of various teleost gonadotropin receptors, has provided the path for more thorough research of these important ligands and their receptors. The current study aimed to gain more insight into the structure and differential specificity of Japanese medaka Fshr (mdFshr) and Lhr (mdLhr) by comparing the activation efficiency with the Nile tilapia Fshr (tiFshr) and Lhr (tiLhr) towards medaka and tilapia gonadotropins. A transactivation assay, followed by amino acid sequence comparison and structural modeling of gonadotropins and receptors from these fish species representing two teleost orders, were performed in order to model the intra- and interspecies ligand-receptor activation. The hormone and receptor biodiversity and cross-species reactivity provide the motivation for a better understanding of hormone specificity, which might unravel key factors of selectivity.

2. Materials and methods

2.1. Animals

Japanese medaka (*Oryzias latipes*) of the d-rR strain were kept in recirculating systems with water temperature of 28 ± 1 °C and light–dark cycle of L14:D10. Embryos were incubated in culture medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, all Sigma-Aldrich, St. Louis, U.S.A.) at 26 °C until hatching and transferred to system tanks. A combination of dry feed and live brine shrimp nauplii (*Artemia salina*) larvae was used to feed the fish three meals per day. Handling, husbandry and use of fish were in accordance with the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences.

2.2. qPCR gene expression analysis of fshr and lhr – Tissue screening

A tissue screening of *fshr* and *lhr* gene expression was performed (brain, pituitary, testis, ovary, eyes, heart, intestine, liver, gills, gallbladder, spleen) according to the methodology described in Burow et al. (2019). Tissues from adult 6 months-old males (n = 3) and females (n = 3) were dissected and total RNA was phenol–chloroform extracted and resuspended in 14 µl nuclease free water. cDNA was prepared from 25 to 500 ng of total RNA (brain 500 ng, pituitary 25 ng, testis 250 ng, ovary 500 ng, eyes 500 ng, heart 100 ng, intestine 500 ng, liver 50 ng, gills 250 ng, gallbladder 100 ng, spleen 500 ng) using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (Thermofisher Scientific). To ensure the presence of a single *fshr* and *lhr* in the medaka genome, we searched for the receptor sequences in the Japanese medaka Hd-rR genome assembly (ASM223467v1) and specific primers were designed, according to Burow et al. (2019) (see Table 1 for qPCR primers sequences). qPCR was performed on a LightCycler 96 Real-Time PCR system (Roche, Mannheim, Germany) using LightCycler

Table 1
qPCR primers used in the present study.

Target	Reference	Primer sequence	Accession number	Amplicon size (nt)	Efficiency
lhr	This study	Forward: 5′-CAGTCTGAGGGGGATTGAAA-3′ Reverse: 5′-AGGAGACGGATGTGAAATCG-3′	ENSORLP00000013664	110	2.04
fshr	This study	Forward: 5′-ATCAGTGGCGATGCCTTCGTGG-3′ Reverse: 5′-CGGGCAAAGAGCTGATGGCTGT-3′	ENSORLP00000039837	79	2.00
18s	Burow et al., 2019	Forward: 5′-CCTGCGGCTTAATTTGACTC-3′ Reverse: 5′-AACTAAGAACGGCCATGCAC-3′	AB105163.1	118	2.02
rpl7	Burow et al., 2019	Forward: 5′-TGCTTTGGTGGAGAAAGCTC-3′ Reverse: 5′-TGGCAGGCTTGAAGTTCTTT-3′	NM_001104870	98	2.03
gapdh	This study	Forward: 5′-CCTCCATCTTTGATGCTGGT-3′ Reverse: 5′- ACGGTTGCTGTAGCCAAACT-3′	XM_004077972.3	75	2.01

480 SYBR Green I Master (Roche). All samples were run in duplicate, each composed of 5 µl mastermix, 5 µM of each of forward and reverse primer, and 3 µl of 10x diluted cDNA sample in a total volume of 10 µl. Cycling parameters of the qPCR were 10 min pre-incubation at 95 °C, 40 cycles of amplification at 95 °C for 10 s, 60 °C for 5 s and 72 °C for 4 or 5 s (4 s for *fshr, rpl7*, and *gapdh,* and 5 s for *lhr* and *18 s*), followed by melting curve analysis to assess qPCR product specificity. Three non-template controls and three positive controls (calibrator) were included in every qPCR plate. The stability of the three candidate reference genes *18 s*, *rpl7* and *gapdh* was evaluated using RefFinder, resulting in the use of *18 s* and *rpl7* as the most stable combination of reference genes. Relative gene expression levels were calculated according to Weltzien et al. (2005) and Hodne et al. (2012).

2.3. qPCR gene expression analysis of fshr and lhr – Testis developmental profile

Testes from 30 males were selected and dissected according to standard body length and age, based on the size groups in Burow et al. (2019): group 1 (14–16 mm, age 46–48 days post fertilization (dpf), group 2 (16–18 mm, age 79–84 dpf), group 3 (18–20 mm, age 100–111 dpf), group 4 (20–22 mm, age 129–134 dpf), group 5 (22–24 mm, age 159–167 dpf), and group 6 (24–26 mm, age 202–208 dpf). Total RNA extraction, cDNA synthesis (from 1000 ng RNA), and qPCR were performed as described above, with the exception that the combination of *18 s*, *rpl7* and *gapdh* was found to be the most stable combination of reference genes and therefore used for normalization of expression data.

2.4. Vector construction of Japanese medaka and Nile tilapia fshr and lhr

Synthesis of full length coding cDNA sequences of medaka *fshr* and *lhr* was outsourced to GenScript (New Jersey, U.S.A.). Two sets of constructs were made for the medaka Fshr and Lhr, one set from the sequences retrieved from the medaka genome, (see section Results 3.1) and another set based on the cloned sequences *fshr* (NM_001201514) and *lhr* (NM_001201515). The open reading frames of both receptors, *fshr* (*mdfshr*) and *lhr* (*mdlhr*), were synthesized and cloned into pcDNA3.1(+) expression vector under the control of the CMV promoter using NheI (5′ end) and HindIII (3′ end) restriction sites. The entire coding regions of Nile tilapia *fshr* (*tifshr*) (AB041762.1) or *lhr1* (*tilhr*) (AB041763.1) were similarly cloned into pcDNA3.1(+), see Aizen et al. (2012b).

2.5. Production of Japanese medaka and Nile tilapia recombinant gonadotropins and pituitary extracts

Recombinant gonadotropins were produced in methylotrophic yeast *Pichia pastoris* cells as single chain polypeptides, according to Burow et al. (2019) (medaka), and Kasuto and Levavi-Sivan (2005) and Aizen et al. (2007) (tilapia). Preparation of Japanese medaka pituitary extract (MPE) or Nile tilapia pituitary extract (TPE) was according to Aizen et al. (2012a), using sexually mature fish. Pituitaries were homogenized on ice in 0.01 M PBS pH 7.0 containing 0.02 M phenylmethylsulfonyl fluoride and 0.05 M EDTA using a Polytron homogenizer. The homogenate was centrifuged at 4 °C, 4100 *g* for 30 min. The pellet was stirred for 30 min to maximize extraction, and centrifuged again under the same conditions. The resulting combined supernatants were used as the pituitary extract. We recently compared several different methods for determination of the recombinant protein concentration, and found that commercial kits that use 6-His-ELISA gave more accurate results than directly measuring absorbance at 280 nm (Hollander-Cohen et al., 2018). The 6-His competitive ELISA was performed according to the manufacturer's instructions (GeneScript) (Hollander-Cohen et al., 2018). Briefly, anti-His monoclonal antibody and His-tagged proteins were added to a microtiter plate, coated with a His-tagged protein. Goat anti-mouse IgG, conjugated with horseradish peroxidase, was used as secondary antibody. The plate was read at 405 nm, using a Spectra II ELISA reader (SLT, Salzburg, Austria). Five serial dilutions of each of the tested recombinant 6-His proteins were measured in duplicate to determine the accurate final concentration of each protein.

2.6. Receptor-*transactivation assay*

The purpose of performing a receptor-transactivation assay was to characterize medaka Fshr and Lhr, and to study intra- and interspecies ligand specificity of gonadotropins comparing medaka and tilapia. Since the main signaling pathway involved in sex steroid production following Fshr and Lhr activation is the cAMP/protein kinase A (PKA), we used a sensitive luciferase (LUC) reporter assay using a vector with cAMP response element (CRE, Invitrogen) to study the activation of medaka and tilapia gonadotropin receptors. Response specificity was validated by activation of the CRE-LUC reporter system by forskolin, an adenylate cyclase activator, whereas 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, was used as negative control (Biran et al., 2008). After 48 h, cells were stimulated with decreasing concentrations (43–0.01 ng/ml for medaka Lhβα/Fshβα and 500–0.12 ng/ml for tilapia Lhβα/Fshβα). Six hours after stimulation, cells were analysed using GloMax-multi detection system (Promega, Madison, USA).

Mammalian COS-7 cells were transfected with both CRE-LUC, and either mdFshr, mdLhr, tiFshr, or tiLhr. For each receptor, activation was tested using recombinant medaka Fshβα (mdFshβα), medaka Lhβα (mdLhβα), Nile tilapia Fshβα (tiFshβα), Nile tilapia Lhβα (tiLhβα), MPE, or TPE. Transient transfection, cell procedures and stimulation protocols were generally according to Levavi-Sivan et al. (2005). Briefly, COS-7 cells were grown in DMEM supplemented with 10% FBS, 1% glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Biological Industries, Israel) under 5% CO_2 until confluent. COS-7 cells (American Type Culture Collection, Rockville, U.S.A.) were co-transfected either with pc-mdLhr (mdLhr cloned into pcDNA3.1 vector), pc-mdFshr, pc-tiLhr, or pc-tiFshr (at 3 µg/plate), together with a reporter

plasmid (CRE-LUC; at 3 µg/ plate), using TransIT-LT1 Transfection Reagent (Mirus, Milton Keynes, U.S.A.). The cells were serum starved for 18 h, stimulated with vehicle of two concentrations of either mdLhβα, mdFshβα, tiLhβα, tiFshβα (140, 550 ng/ml), or diluted pituitary extracts (1:100 for both MPE and TPE) for 6 h, and then harvested and analyzed with Lumac Biocounter M2500 (Celsis, The Netherlands). Harvested COS-7 cell were lysed in 35 µl lysis buffer (Biological industries, Kibbutz Beit-Haemek, Israel) and assayed for LUC activity by assaying 10 µl with buffer containing luciferin and ATP for LUC activity. The data were normalized to the number of COS-7 cells in each plate. Transfection experiments were performed in triplicate with three independently isolated sets. COS-7 cells, transfected with a vector without gonadotropin receptor cDNA, showed no change in LUC activity in any experimental group (data not shown). EC50 values were calculated from dose response curves by means of computerized nonlinear curve fitting on baseline-corrected (control) values using Prism version 6 software (GraphPad).

2.7. Sequence comparison of gonadotropin β-subunits and receptors

To better understand the medaka gonadotropin receptor function, we compared amino acid sequences of medaka gonadotropin β-subunits and gonadotropin receptors with their homologous sequences of gonadotropin β-subunits and receptors of human and four other teleosts, including two other Acanthopterygii; the Nile tilapia and the European seabass. Gene sequences were retrieved from Genbank, and deduced amino acid sequences were aligned using CLC Main workbench (Qiagen) and a previous alignment as template for the gonadotropin receptors (Maugars and Dufour, 2015). Signal peptide and N-glycosylation sites were predicted using CLC Main workbench. Tyrosine sulfation sites were predicted using Sulfosite browser with 80% prediction sensitivity (Chang et al., 2009).

2.8. Structural models of gonadotropin β-subunits, α-subunits and receptors

Structural modeling of medaka and tilapia gonadotropin β-subunits, α-subunits and receptors was performed to analyze and predict ligand-receptor binding and activation. Sequence alignment was carried out using *EMBOSS*-NEEDLE Pairwise sequence alignment tool (Rice et al., 2000). 3-D structures for medaka and tilapia gonadotropin β-subunits, α-subunits, and gonadotropin receptors were developed using I-Tasser online tool (a bioinformatics method for predicting 3D structural models of protein molecules (Roy et al., 2010; Yang et al., 2015; Zhang, 2008)) with the available sequences. Protein models were further rendered and prepared using Maestro tool in Schrödinger software. Structural alignment and superposition were done using Schrödinger Maestro (Schrödinger Release 2018-3) and verified using PyMOL (PyMOL, Molecular Graphics System, version 2.0 Schrödinger). Structural superposition was performed using alpha carbon chain of the molecules. The root mean square deviation (RMSD) of atomic positions was calculated as a parameter of the divergence of two aligned structures from each other, hence to assess their structural similarity.

2.9. Statistical **analysis**

Data are presented as mean ± SEM. qPCR data were tested for normal distribution by the Shapiro-Wilk normality test. For normally distributed data, potential significance of differences in expression levels between body length groups was calculated by one-way analysis of variance (ANOVA), followed by Tukeýs multiple comparison test (post-hoc test) using Graph-Pad Prism software (version 7; GraphPad, San Diego, U.S.A.). For sample groups that did not follow a normal distribution, the data were first log-transformed. Data from the receptor transactivation assay were tested in the same manner, but using JMP software (version 10; North Carolina, U.S.A.). All data of the correlation analysis between gene expression levels were first tested for normality by means of D'Agostino-Pearson normality test. Since the datasets were normally distributed, Pearson correlation coefficients were calculated for all analyses. Significance level was set to 0.05.

3. Results

3.1. Medaka ***fshr and lhr gene identification***

A single *fshr* gene (ENSORLP00000039837) and a single *lhr* gene (ENSORLP00000013664) were identified in the new medaka genome assembly. The CDS encoding for medaka *fshr* and *lhr* are 2064 bp and 2067 bp, respectively, and they share 99.95% and 99.52% nucleotide identity, respectively, with the medaka *fshr* and *lhr* cloned sequences (Ogiwara et al., 2013). The base differences encode for different amino acids in both receptors. For *fshr*, an amino acid substitution from cysteine (in the medaka genome) to tyrosine (cloned cDNA by Ogiwara et al., 2013) is present in the transmembrane domain at position 618. For *lhr*, four amino acid substitutions are present between the genome sequence and the cloned sequence (A5 versus V13, D42 versus G50, S304 versus G312 and F623 versus S631).

3.2. qPCR gene expression analysis of fshr and lhr – Tissue screening

Both *fshr* and *lhr* were found to be highly expressed in ovary and testis of adult female and male medaka, respectively (Fig. 1). *fshr* was also found to be expressed at low levels in brain, and at trace amounts in eyes, gills, gallbladder, intestine, spleen and pituitary. *fshr* was not detected in heart and liver (Fig. 1A). High non-gonadal expression of *lhr* was revealed in brain. Low levels of *lhr* were observed in eyes, and trace amounts in gills, gallbladder, heart, intestine, spleen and pituitary (Fig. 1B). No *lhr* expression was detected in liver.

3.3. ***qPCR gene** expression **analysis of fshr and lhr – Testis developmental profile***

The *fshr* expression data for the developmental profile targeting six groups of increasing standard body length ranges were not normally distributed, and thus were log-transformed. Significant differences in *fshr* expression were detected between males of body length 14–16 mm (group 1) and 18–20 mm (group 3), between 16 and 18 mm (group 2) and 18–20 mm (group 3), and between 16 and 18 mm (group 2) and 24–26 mm (group 6) ($p < 0.05$) (Fig. 2A), with the highest statistical difference between group 2 and 3. The *lhr* expression data were normally distributed for all size groups. A significant difference of *lhr* expression was detected between body length 14–16 mm (group 1) and 18–20 mm (group 3), and between 16 and 18 mm (group 2) and 18–20 mm (group 3) ($p < 0.05$) (Fig. 2B). Both *fshr* and *lhr* showed lowest expression levels in group 2, and highest in group 3. There was a strong positive correlation between *fshr* and *lhr* expression levels during gonadal development (Pearson correlation coefficient $r = 0.9751$, $p < 0.0009$).

3.4. Receptor-transactivation assay

Effects of recombinant gonadotropins from medaka (mdLhβα, mdFshβα) and tilapia (tiLhβα, tiFshβα) or diluted pituitary extracts from medaka (MPE, 1:10) and tilapia (TPE, 1:100) on the cognate receptors (Fshr and Lhr) from both species were investigated. The functionality of the receptors was studied by transient transfection assays in COS-7 cells, expressing one of the receptors at the time. Each of the medaka recombinant gonadotropins, mdFshβα and mdLhβα, were able to activate its own cognate receptor. Recombinant mdFshβα significantly stimulated CRE-LUC activity in COS-7 cells expressing the mdFshr in a dose-dependent manner (EC50 = 0.29 ± 0.03 ng/ml) (Table 2, Fig. 3A). Furthermore, mdFShβα activated mdLhr in a dose-dependent manner (EC50 = 0.64 ± 0.19 ng/ml) (Table 2, Fig. 3B).

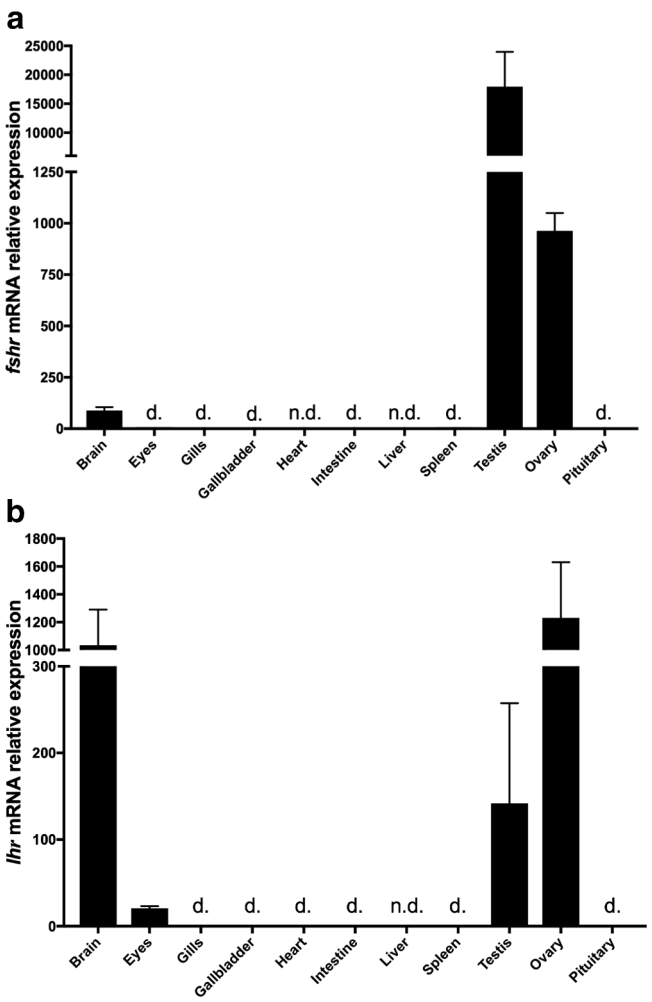

Fig. 1. Tissue distribution of *fshr* and *lhr* transcripts in medaka. Messenger RNA levels for *fshr* (A) and *lhr* (B) were assayed by qPCR in various tissues of adult medaka: brain, eyes, gills, gallbladder, heart, intestine, liver, spleen, testis, ovary, and pituitary (n = 3). d., detected but not quantifiable, nd., non-detected. Data are given as mean ± SEM.

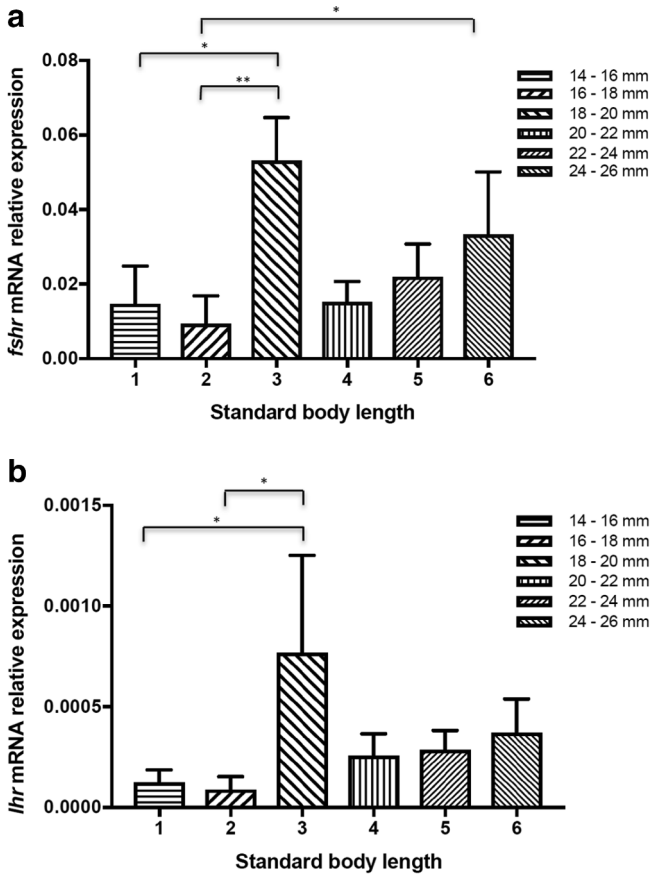

Fig. 2. Developmental profile of testicular *fshr* and *lhr* gene expression in male medaka. Transcript levels for *fshr* (A) and *lhr* (B) were measured in medaka classified according to standard body length (group 1 14–16 mm, group 2 16–18 mm, group 3 18–20 mm, group 4 20–22 mm, group 5 22–24 mm, group 6 24–26 mm) (n = 5). Means marked by asterisks differ significantly (*p ≤ 0.05, ** p ≤ 0.01).

Concerning the mdLhβα, it stimulated CRE-LUC activity in COS-7 cells expressing mdLhr (EC50 = 1.73 ± 0.16 ng/ml) (Tab. 3 Fig. 3B), and to a lower extent activated cells expressing the mdFshr (EC50 = 5.69 ± 0.24 ng/ml) (Table 2, Fig. 3A).

Interestingly, the tiLhr was efficiently activated by mdLhβα (EC50 = 0.47 ± 0.05 ng/ml) (Table 2, Fig. 3D) and mdFshβα (EC50 = 1.38 ± 0.09 ng/ml) (Table 2, Fig. 3D) in a dose-dependent manner. In addition, mdFshβα generated a concentration-dependent increase in the activation of tiFshr (EC50 = 0.33 ± 0.10 ng/ml) (Table 2, Fig. 3C). tiFshr was also shown to be activated by mdLhβα, however to a lower extent than mdFshβα, (EC50 = 6.44 ± 0.11 ng/ml) (Table 2, Fig. 3C).

Tilapia gonadotropins, tiFshβα and tiLhβα, could both activate mdFshr in a dose-dependent manner (EC50 = 11.2 ± 0.14 ng/ml and 2.54 ± 0.06 ng/ml, respectively; Table 2, Fig. 4A). Furthermore, both tiFshβα and tiLhβα could activate mdLhr, but to a much lower extent and with lower EC50s compared to mdFshr (EC50 = 1.3 x 10^{-5} ± 0.09 ng/ml and 0.058 ± 0.06 ng/ml, respectively; Table 2, Fig. 4B).

MPE significantly activated the mdLhr (Fig. 5A), while MPE did not stimulate CRE-LUC activity in COS-7 cells expressing the mdFshr (Fig. 5B). However, TPE significantly activated both the mdLhr (Fig. 5C) and mdFshr (Fig. 5D). MPE and TPE stimulated CRE-LUC activity in COS-7 cells expressing either tiLhr (Fig. 5E) or tiFshr (Fig. 5F).

Table 2
Effective half-maximum concentration (EC_{50}, ng/ml) calculated for the medaka and tilapia Fsh and Lh receptors, Fshr and Lhr. A CRE-LUC reporter gene was used to quantify the activation of the PKA pathway. Results are presented as mean ± SEM (n = 3).

	mdFshr	mdLhr	tiFshr	tiLhr
mdFshβα	0.29 ± 0.03	0.64 ± 0.19	0.33 ± 0.1	1.38 ± 0.09
mdLhβα	5.69 ± 0.24	1.73 ± 0.16	6.44 ± 0.11	0.47 ± 0.05
tiFshβα	11.2 ± 0.14	1.3 x 10^{-5} ± 0.09		
tiLhβα	2.54 ± 0.06	0.058 ± 0.06		

3.5. Sequence comparison of gonadotropin β-subunits and receptors

To rationalize the activation profile of gonadotropin receptors by the different ligands, the deduced amino acid sequences of medaka Fshβ and Lhβ were first compared with homologous subunits of human and four other teleosts, including two other Acanthopterygii, the Nile tilapia and the European seabass. Sequence comparison showed a higher divergence between the medaka and tilapia Fshβ (59% identity, 71.8% similarity) than the medaka Lhβ and tilapia Lhβ (66.7% identity, 80.3% similarity). Based on alignment of cysteine residues, medaka and tilapia Lhβ conserved the twelve cysteines common to glycoprotein hormone β-subunits, while Fshβ of medaka, contrary to most other Acanthopterygii, conserved the position of only 11 cysteine residues

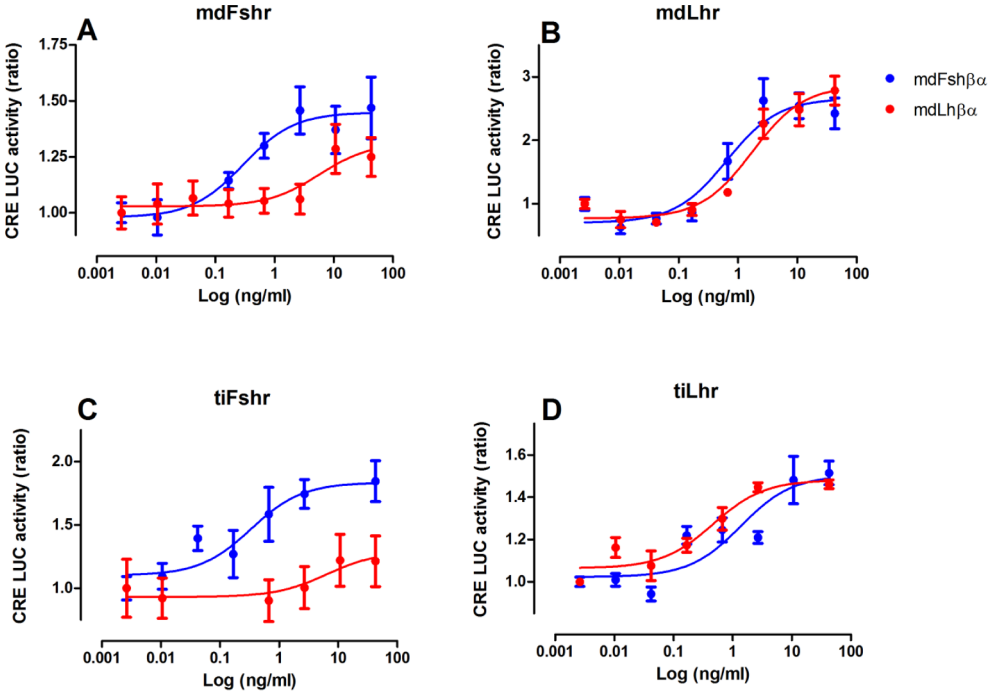

Fig. 3. Receptor-transactivation assay of medaka and Nile tilapia gonadotropin receptors for characterization of intra- and interspecies ligand selectivity using homologous and heterologous ligands. Medaka Fshβα and Lhβα induced transcriptional activity in COS-7 cells that were co-transfected with medaka Fshr (mdFshr) (A), medaka Lhr (mdLhr) (B), Nile tilapia Fshr (tiFshr) (C), or Nile tilapia Lhr (tiLhr) (D) together with a CRE-LUC reporter plasmid. Cells were treated with various concentrations of medaka Fshβα and Lhβα (highest concentration 43 ng/ml; serial dilutions of 1:4). The data are expressed as change in luciferase activity over basal levels, and data from one of a total of three experiments are shown. Each ligand concentration was run in triplicate per experiment and is shown as mean ± SEM.

(Fig. 6). In medaka Fshβ, the third cysteine is shifted three residues downstream, whereas it has been lost in both tilapia and European seabass Fshβ. The latter two instead have an additional cysteine upstream of cysteine C1. Medaka and the other acanthopterygian Fshβ show shorter length compared to Atlantic salmon Fshβ, European eel (*Anguilla anguilla*) Fshβ, and human FSHβ, including several amino acid deletions between C1 and C2, C5 and C7, and C7 and C8. For both medaka Fshβ and Lhβ, a single N-glycosylation site is present, positioned between C1 and C2 for medaka Fshβ, and between C10 and the C11 for medaka Lhβ (Fig. 6).

Alignment of gonadotropin receptors showed that medaka Fshr and Lhr both conserved the typical structure of gonadotropin receptors with a long ECD including a conserved LRR region connected to the transmembrane domain by a hinge region highly variable in amino acid sequence composition and length. Comparison of the primary structure revealed a lower conservation between the medaka and tilapia Fshr (61.9% identity, and 71.1% similarity) than between medaka and tilapia Lhr (76.6% identity, and 85.7% similarity) (Fig. 7).

The ECD in medaka Lhr consisted of 11 successive conserved LRRs, as in other vertebrate Lhrs, while an additional LRR to the 11 LRRs was found positioned between the LRR2 and LRR3 in medaka Fshr. This extra LRR was also observed in Nile tilapia and European seabass Fshr. Three and four N-glycosylation sites were predicted for medaka Fshr and Lhr, respectively. Among these N-glycosylation sites, both medaka Fshr and Lhr showed the site on LRR7 common to all glycoprotein hormone receptors. Medaka Fshr shared the 3 N-glycosylation sites with the Nile tilapia Fshr. The two first N-glycosylation sites of the medaka Lhr were conserved in Nile tilapia Lhr1. In contrast, no tyrosine sulfation site could be predicted in either medaka or tilapia Fshr or Lhr (Fig. 7). The transmembrane domain (TM) of both Fshr and Lhr showed a high amino acid identity between orthologous receptors, in particular in TM2, TM3, TM6 and TM7, except for the conserved cysteine residue of TM6 that has been substituted by a glycine G581.

3.6. *Structural* ***models of gonadotropin β-subunits, α-subunits and receptors***

Since mechanisms of biological activity of a molecule are highly dependent on its structure, structural modeling of medaka and Nile tilapia gonadotropin β-subunits, α-subunits and receptors was performed. The generated structures for medaka Fshβ (Fig. 8A) and Nile tilapia Fshβ (Fig. 8B), as well as medaka Lhβ (Fig. 8E) and Nile tilapia Lhβ (Fig. 8F), and their receptors Fshr (Fig. 8I-J) and Lhr (Fig. 8M-N), which all belong to the GPCR family, were screened and selected on the basis of high confidence score and stability. They were further prepared by processing the structures for structural alignment and superposition. A high structural similarity was observed between the predicted models for the β-subunits of both Fsh and Lh. The β sheet domains were seen to

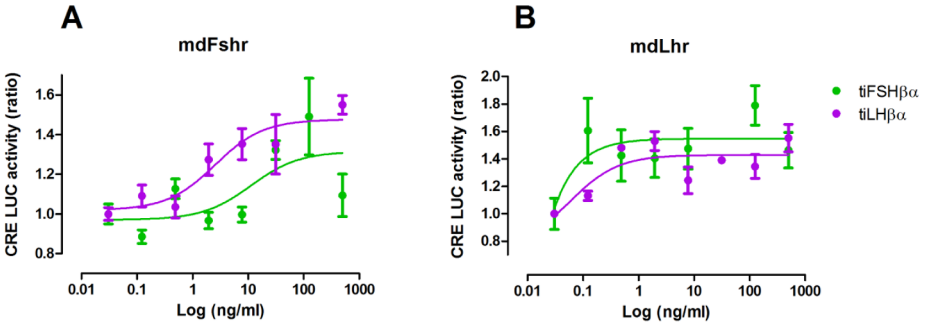

Fig. 4. Receptor-transactivation assay of medaka gonadotropin receptors for characterization of interspecies ligand specificity using Nile tilapia ligands. Nile tilapia Fshβα and Lhβα induced transcriptional activity in COS-7 cells that were co-transfected with medaka Fshr (mdFshr) (A), medaka Lhr (mdLhr) (B) together with a CRE-LUC reporter plasmid. Cells were treated with various concentrations of medaka Fshβα and Lhβα (highest concentration 500 ng/ml; serial dilutions of 1:4). The data are expressed as change in luciferase activity over basal levels, and data from one of a total of three experiments are shown. Each ligand concentration was run in triplicate per experiment and is shown as mean ± SEM.

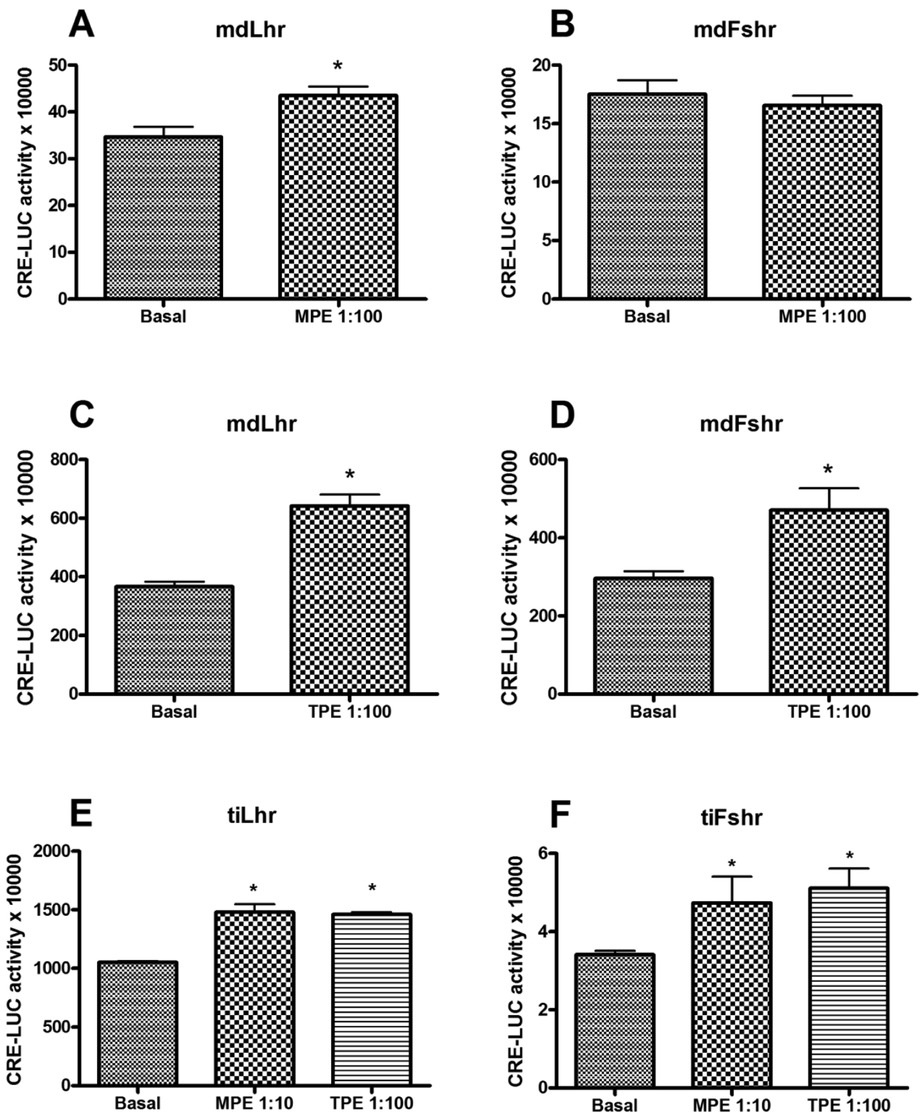

Fig. 5. Receptor-transactivation assay of medaka and Nile tilapia gonadotropin receptors for characterization of intra- and interspecies ligand specificity using medaka and Nile tilapia pituitary extracts. COS-7 cells were transiently co-transfected with medaka gonadotropin receptors (mdFshr, mdLhr) (A-D) or tilapia gonadotropin receptors (tiFshr, tiLhr) (E-F), each together with a CRE-LUC reporter plasmid. Cells were stimulated with medaka pituitary extract (MPE; 1:10) (A, B, E, F), or tilapia pituitary extract (TPE; 1:100) (C, D, E, F). Each assay was repeated more than three times, and each treatment was conducted in triplicates. Data (mean ± SEM) are expressed as the increase in luciferase activity over basal activity. Means marked with different letters and means marked by asterisks differ significantly, $p \leq 0.05$.

be highly conserved between medaka Fshβ (Fig. 8A) and Nile tilapia Fshβ (Fig. 8B), as well as between medaka Lhβ (Fig. 8E) and Nile tilapia Lhβ (Fig. 8F). Superposition of both gonadotropin β-subunits revealed structural homology between the Fshβ of both medaka and Nile tilapia (Fig. 8C), and between Lhβ of medaka and Nile tilapia (Fig. 8G). Structure alignment between medaka and Nile tilapia Fshβ (Fig. 8D), and between Lhβ of medaka and Nile tilapia (Fig. 8H) detected high similarity in β sheet domains. The α-subunit region, which is common for both hormones within the species, displayed a varying homology between medaka (Fig. 8Q) and Nile tilapia (Fig. 8R). The structural alignment revealed a similar but non-optimally aligned structure (Fig. 8S).

```
                                                         1                  2  3          4    5
Human FSHβ      -----------------MKTLQFFFLFCCWKAICCNSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDP  60
Eel Fshβ        -------------MHLAVTALCLTLAPILARA--STSCGLANISISVENEECGGCVTFNTTACAGLCFTQDSVYKSS  62
Salmon Fshβ     ------MYCTRLRTLQLVVMATLWVTPVRAGTDCRYGCRLNNMTITVEREDCHG--SITITTCAGLCETTDLNYQST  69
Tilapia Fshβ    ---------------MQLVVMAAVLALAGAEQDCSSGCRPKNISL--PVDTCGF---VDTTICEGQCFQKDPNFIHT  57
Medaka Fshβ     ---------------MQLVVMAAALVLAEVGQVSSFSCHPKNVSI--PVESCGISGCVHTTICEGRCYHEDPNYISY  60
Seabass Fshβ    ---------------MQLVVMVAVLALARAGQGCSFGCHPTNISI--QVESCGLTEVIYTTICEGQCYHEDLVYLSH  60
Human LHβ       ---------MEMLQGLLLLLLLSMGGAWASREPLRPWCHPINAILAVEKEGCPVCITVNTTICAGYCPTMMRVLQAV  68
Eel Lhβ         --------MSVYPECTWLLFVCLCHLLVSAGGSLLLPCEPINETISVEKDGCPKCLVFQTSICSGHCITKDPSYKSP  69
Salmon Lhβ      --------MLGLHVGT-LISLFLCILLEPVEGSLMQPCQPINQTVSLEKEGCPTCLVIQTPICSGHCVTKEPVFKSP  68
Tilapia Lhβ     MMAQISRMLLALMLSLFVGASTFI--LSPAAAFQLPPCQLINQTVSLEKEGCPSCHPVETTICSGHCITKDPVIKIP  75
Medaka Lhβ      MISRVSRVMFLLMLSFILGTSTFLWSLAPAAAFQLPYCQPVKQKLSLQKEGCSGCHTVETTVCSGHCLTKDPLMKI-  76
Seabass Lhβ     MAVQASRVMFPLVLSLFLGATSSIWPLATAEAFQLPPCQLINQTVSLEKEGCPKCHPVETTICSGHCITKDPVIKIP  77

                        6              7                8 9 10       11         12
Human FSHβ      ARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE----- 129
Eel Fshβ        LKPYPQQACNFRDVVYETVHLPGCPSGMDLHFTYPVALSCECSKCNTDSTDCGPLNTEVSGCLTH--------- 127
Salmon Fshβ     WLPRSQGACNFKDWSYEKVYLEGCPSGVDP-FFIPVAKSCDCIKCKTDNTDCDRISMATPSCLGSPLEM----- 137
Tilapia Fshβ    DDWPKQKTCN-GEWSYEVKYTEQCPRG----FIYPVARKCECTACNA-NTDCGTLSGYIPSC------------ 113
Medaka Fshβ     EDHPKEKICS-GDWSYEVKFIEGCPVG----FKYPVAKSCECTTCNTRTTYCGRLSADMPSC------------ 117
Seabass Fshβ    YERPEQRICN-GDWSYEVKHIKGCPVG----VTYPVARNCECTTCNTENTDCGRFPGDIPSCLSF--------- 120
Human LHβ       LPPLPQVVCTYRDVRFESIRLPGCPRGVDPVVSFPVALSCRCGPCRRSTSDCGGPKDHPLTCDHPQLSGLLFL- 141
Eel Lhβ         LSTVYQRVCTYRDVRYETVRLPDCRPGVDPHVTFPVALSCDCNLCTMDTSDCAIQSLRPDFCMSQRAS-L-PA- 140
Salmon Lhβ      FSTVYQHVCTYRDVRYETIRLPDCPPWVDPHVTYPVALSCDCSLCNMDTSDCTIESLQPDFCITHRALMDGNMW 142
Tilapia Lhβ     FSNVYQHVCTYRDLYYKTFELPDCPPGVDPIVTYPVALSCHCGRCAMDTSDCTFESMQPDFCMNDIPF---YY- 145
Medaka Lhβ      RSIQYQNVCTYRDFYYKTFELPDCLPGVDPSVTYPVALSCHCGACIMNASDCTFESLPPDFCVKHDSF---YY- 146
Seabass Lhβ     FSNVYQHVCTYRNSHYKTFELPDCPPGVDPTVTYPVAQSCHCGRCAMDTSDCTFESLQPNFCMNDIPF---YY- 147
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Fig. 6. Amino acid sequence alignment of medaka Fshβ and Lhβ with other teleost representatives as well as with the human FSHβ and LHβ sequences. Residues of the signal peptide sequence are in italics. Conserved cysteine residues are numbered in red and other conserved residues are indicated in blue. Putative N-glycosylation sites are labeled in green. The sequence accession numbers are: human *FSHB* (NG_008144), European eel *fshb* (AY169722), Atlantic salmon *fshb* (LOC100136362), Nile tilapia *fshb* (LOC100534500), medaka *fshb* (NM_001309017.1), European seabass *fshb* (AF543314), human *LHB* (LOC3972), European eel *lhb* (X61039), Atlantic salmon *lhb* (LOC10038038), Nile tilapia *lhb* (LOC100534501), medaka *lhb* (AB541982.1), European seabass *lhb* (AF543315).

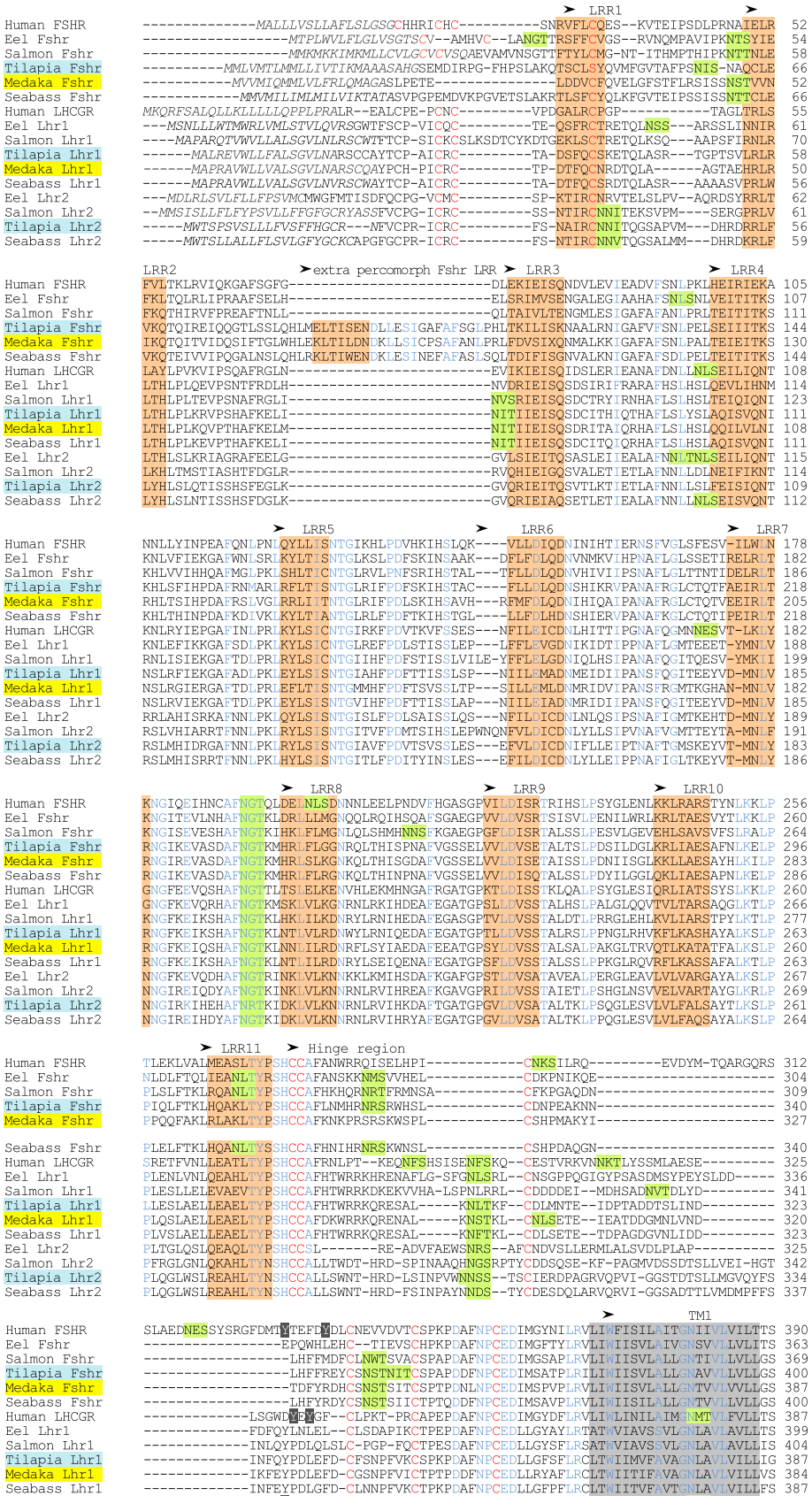

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Fig. 7. Amino acid sequence alignment of medaka Fshr and Lhr with those of other teleost representatives as well as with the human Fshr and Lhr sequences. Residues of the signal peptide sequence are in italics. Leucine rich motifs are highlighted in orange. Arrows indicate the beginning of the different parts of the extracellular domain. Conserved cysteine residues are indicated in red and other conserved residues in blue. Putative N-glycosylation sites are highlighted in green and potential tyrosine sulfation sites are boxed in grey. The sequence accession numbers are: human FSHR (P23945), European eel *fshr* (LN831181), Atlantic salmon *fshr* (NM_001123610.1), Nile tilapia *fshr* (LOC100534395), Japanese medaka *fshr* (ENSORLP00000039837), European seabass *fshr* (FQ310507), human *LHCGR* (NP_000224), European eel *lhr1* (LN831182), Atlantic salmon *lhr1* (LOC106589743), Nile tilapia *lhr1* (LOC100534397), Japanese medaka *lhr* (ENSORLP00000013664), European seabass *lhr1* (HG916828), European eel *lhr2* (LN831183), Atlantic salmon *lhr2* (LOC100136406), Nile tilapia *lhr2* (LOC100705740), European seabass *lhr2* (AY642114.1/HG916828).

Superposition of Lhr displayed a higher structural homology between both species (Fig. 8O), in contrast to Fshr (Fig. 8K). Similarly, structure alignment between Lhr of these species (Fig. 8P) displayed high similarity in transmembrane regions, compared to Fshr (Fig. 8L). A very good structural alignment was observed between Lhr of the two species (Fig. 8P). The structural alignment between the Fshr of medaka and Nile tilapia was observed to lack the similarity needed for a meaningful alignment (Fig. 8L). In addition, the receptors were revealed to be structurally similar comparing medaka Fshr (Fig. 8I) and Nile tilapia Fshr (Fig. 8J), as well as medaka Lhr (Fig. 8M) and Nile tilapia Lhr (Fig. 8N). Structure alignment and superposition have been verified using structural overlapping for medaka and Nile tilapia Fshβ (supplementary data Fig. S1a), medaka and Nile tilapia Lhβ (supplementary data Fig. S1b), medaka and Nile tilapia Fshr (supplementary data Fig. S1c), and medaka and Nile tilapia Lhr (supplementary data Fig. S1d).

The sequence alignment revealed a sequence identity between medaka and Nile tilapia Fshβ (Fig. 6) of 59%, and a similarity of 71.8%. The alignment score of the structural alignment was shown to be 0.121, and the RMSD value 1.74 Angstrom. Comparing medaka and Nile tilapia Lhβ (Fig. 6), the sequence identity was observed to be 66.7%, the sequence similarity 80.3%, the alignment score of the structural alignment 0.474, and the RMSD value 3.44 Angstrom. The sequence alignment detected a sequence identity between medaka and Nile tilapia Fshr (Fig. 7) of 61.9%, and a similarity of 71.1%. The alignment score of the structural alignment was reported to be 1.06, and the RMSD value 5.12 Angstrom. For Lhr (Fig. 7), the sequence identity between medaka and Nile tilapia was observed to be 76.6%, and the sequence similarity 85.7%. The alignment score of the structural alignment was calculated to be 0.507, and the RMSD 3.56 Angstrom.

In conclusion, molecular determinants that dictate the level of interaction specificity were identified by providing a structural view of the interactions.

4. Discussion

In this study we have characterized medaka gonadotropin receptors, Fshr and Lhr, including i) qPCR tissue screen of gene expression; ii) testicular gene expression profile during sexual maturation; iii) activation of medaka Fshr and Lhr using recombinant medaka Fsh and Lh, and medaka pituitary extract; and iv) characterization of the intra- and inter-species ligand specificities of gonadotropins from medaka and Nile tilapia. Fish provide excellent models to investigate species specificity of gonadotropins to their cognate and diverse receptors, due to their rapid molecular evolution (Albertson and Kocher, 1999).

The tissue screen revealed that medaka *fshr* and *lhr* were highly expressed in ovary in female and testis in male, in 6 months-old adult medaka. This was expected and is in accordance with previous studies (e.g. Maugars and Schmidt, 2006 (Atlantic salmon); Kazeto et al., 2012 (Japanese eel); Maugars and Dufour, 2015 (European eel)), underlining the importance of both glycoprotein hormones in the regulation of gonadal development. Non-gonadal *fshr* expression in the present study was detected at low levels in brain, and at trace amounts in eyes, gills, gallbladder, intestine, spleen and pituitary in medaka. Non-gonadal *lhr* expression was observed at high levels in brain, at low levels in eyes, but only at trace amounts in gills, gallbladder, heart, intestine, spleen and pituitary. Even though the expression of *fshr* and *lhr* is found mainly in the gonads, *fshr* and *lhr* transcripts have been observed in non-gonadal tissues. *fshr* transcripts were revealed in kidney of zebrafish (Kwok et al., 2005), and in brain of Atlantic salmon (Andersson et al., 2009), Atlantic halibut (*Hippoglossus hippoglossus*) (Kobayashi et al., 2008), and European eel (Maugars and Dufour, 2015). Gene expression of *fshr* was shown at low abundance in gills of Atlantic salmon (Maugars and Schmitz, 2006), European eel (Maugars and Dufour, 2015), and Senegalese sole (Chauvigné et al., 2010), in accordance with the results of the present work. Gene expression of *lhr* was shown in head kidney of African catfish (Kumar et al., 2001a; Vischer et al., 2003) and European seabass (Rocha et al., 2007a), and in brain of European seabass (*lhr2;* Rocha et al., 2007a), African catfish (*lhr2;* Vischer et al., 2003), Atlantic salmon (*lhr2;* Maugars and Schmitz, 2006), and Atlantic halibut (*lhr1;* Kobayashi et al., 2008). The observation of *lhr* transcripts in brain corresponds to the results of the present study. Furthermore, *lhr* transcripts were shown at low levels in gills and very low levels in heart and liver of Atlantic salmon (*lhr2;* Maugars and Schmitz, 2006), revealing overlapping results with the present study regarding gills and heart. *lhr* gene expression was shown in gills of channel catfish (*lhr1;* Kumar et al., 2001b), Atlantic halibut (*lhr1*; Nyuji et al., 2013), and European seabass (*lhr2*; Rocha et al., 2007b), and in the eye of European eel (*lhr1* and *lhr2*; Maugars and Dufour, 2015) and European seabass (*lhr2;* Rocha et al., 2007b). The expression of *fshr* and *lhr* in gills suggests that gonadotropin receptors might participate in the complex multi-endocrine regulation of gill function in teleosts (Maugars and Dufour, 2015). The expression of *lhr1* and *lhr2* in eye of the European eel may reflect an ancestral function of this organ, which might have been conserved by both duplicated receptors (Maugars and Dufour, 2015). In human, *LHR* transcripts and LHR have been determined in retinal photoreceptors, where the receptor is suggested to be involved in local modulation of visual stimuli (Dukic-Stefanovic et al., 2012). Interestingly, *LHR* expression has been observed in the brain of amphibians (Yang et al., 2007), birds (You et al., 2000), and mammals including human (Lei et al., 1993). In mammals, several functions for brain LHR have been proposed, like GnRH regulation (Hu et al., 2006), sensory modulation, or fetal neurogenesis (Al-Hader et al., 1997). Because *lhr* is highly expressed in medaka brain, as in some other teleosts, investigations on the potential function of gonadotropin signaling in the vertebrate brain should be performed in future studies.

Both Fshr and Lhr were expressed in all testis stages, from immature to fully mature. This is in agreement with Baron et al. (2005) showing that *fshr* of rainbow trout is already expressed before sex differentiation and the presence of both gonadotropin receptors in Atlantic salmon and European seabass in immature testis in juvenile males (Maugars and Schmitz, 2008; Rocha et al., 2009). Data on the expression of gonadotropin receptor genes in male fish are mainly focused on seasonal spawners, and still limited for daily spawners, such as medaka. The developmental gene expression profile in the present study detected a significant increase of *fshr* mRNA levels from body length group 1 (14–16 mm) to 3 (18–20 mm), from group 2 (16–18 mm) to 3, and from group 2 to group 6 (24–26 mm). A significant increase of *lhr* transcripts was revealed from body length group 1 to 3, and from group 2 to 3. Our previous testis histology study revealed that male medaka between 14 and 19 mm include individuals at different developmental stages, from

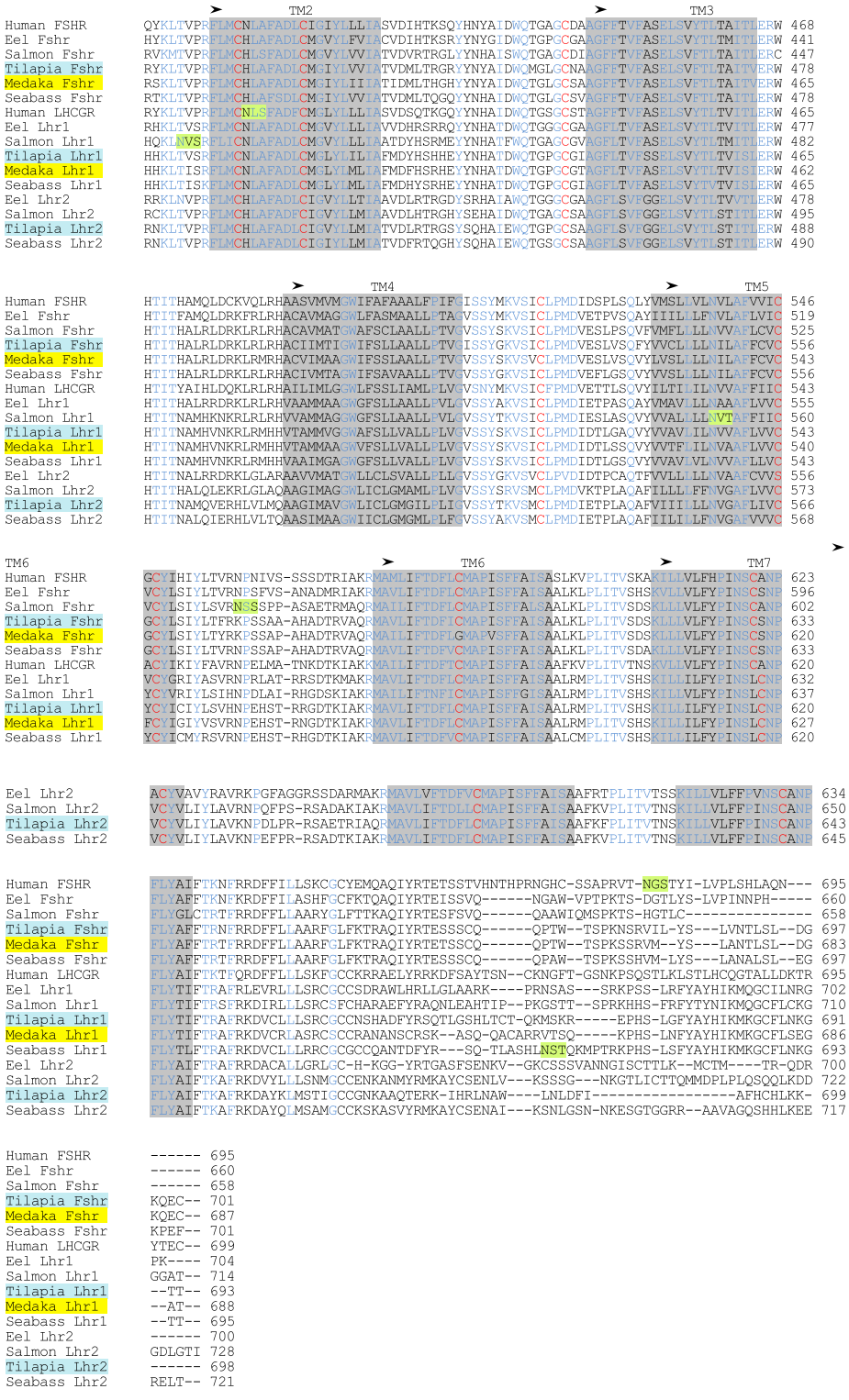

Fig. 7. (*continued*)

immature with only spermatogonia, to fully mature with spermatozoa, and that males above 19 mm were sexually mature (Burow et al., 2019). The results showed that *fshr* and *lhr* mRNA levels increased during the transition leading to fully mature testis, in concordance with various studies. In salmonids, *fshr* upregulation during early spermatogenesis was associated to Sertoli cell proliferation occurring concomitantly with spermatogonial proliferation (Baron et al., 2005; Maugars and Schmitz, 2008). In male yellowtail and rainbow trout, *fshr* levels were shown to increase during early spermatogenesis, whereas at spermiation, mRNA levels decrease in yellowtail and fluctuate for rainbow trout (Rahman et al., 2003; Kusakabe et al., 2006). For rainbow trout, Atlantic salmon, and yellowtail males, *lhr* mRNA levels were shown to steadily increase as testicular maturation advances (Rahman et al., 2003; Kusakabe et al., 2006; Maugars and Schmitz, 2008). Interestingly, a correlation analysis revealed a strong positive correlation between *fshr* and *lhr* mRNA expression profile in medaka. The peak at body length 18–20 mm for both *fshr* and *lhr* transcript level shown in this study could result from a higher number of males

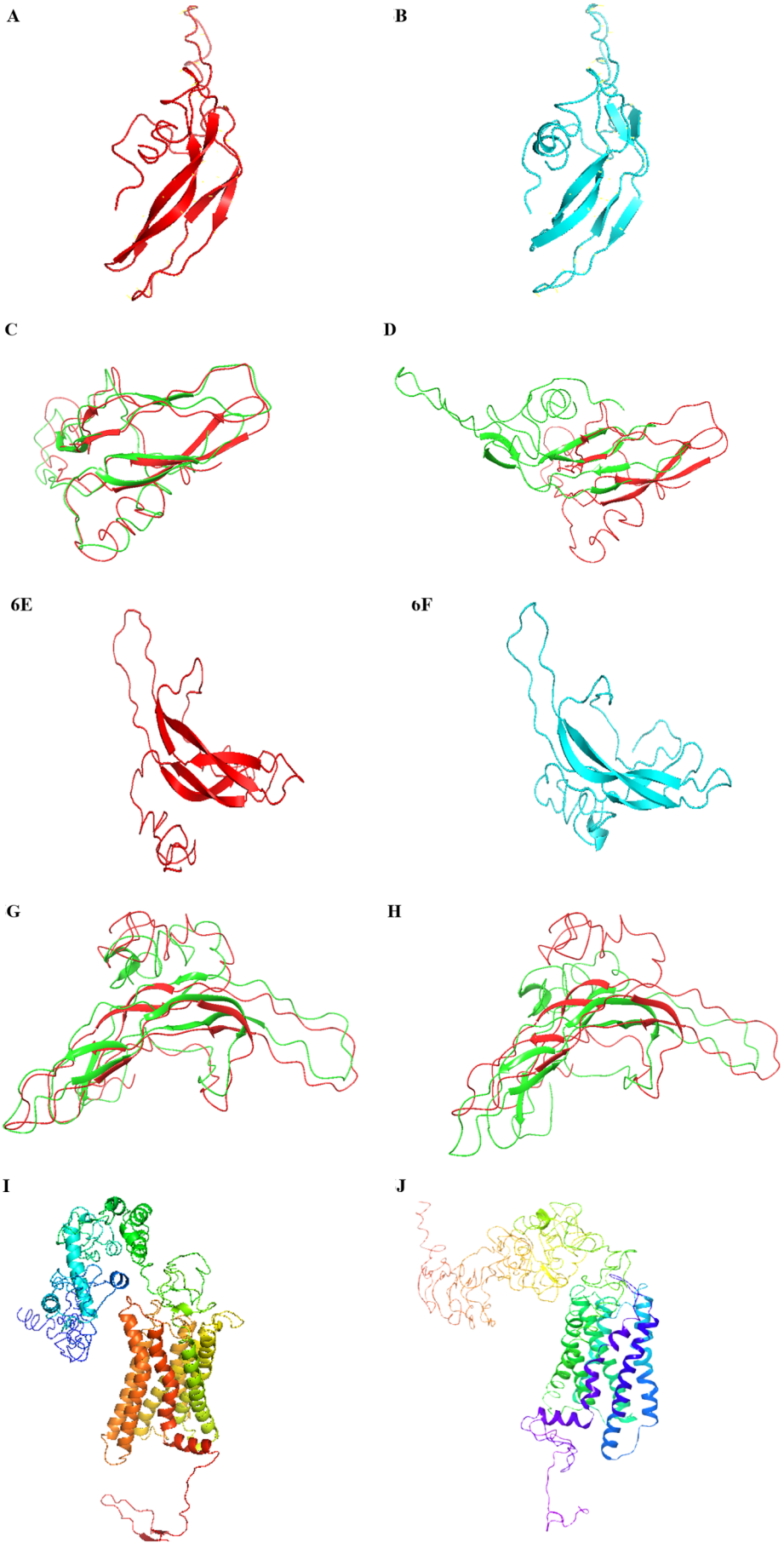

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Fig. 8. Medaka and Nile tilapia gonadotropin β-subunit, α-subunit and gonadotropin receptor models. Structural three-dimensional model of medaka Fshβ (A), tilapia Fshβ (B), medaka Lhβ (E), tilapia Lhβ (F), medaka Fshr (I), tilapia Fshr (J), medaka Lhr (M), tilapia Lhr (N), medaka alpha subunit (Q), and tilapia alpha subunit (R). Superposition of medaka Fshβ (red) with tilapia Fshβ (green) (C), medaka Lhβ (red) with tilapia Lhβ (green) (G), medaka Fshr (blue) with tilapia Fshr (red) (K), and medaka Lhr (blue) with tilapia Lhr (red) (O). Structure Alignment of medaka Fshβ (red) with tilapia Fshβ (green) (D), medaka Lhβ (red) with tilapia Lhβ (green) (H), medaka Fshr (blue) with tilapia Fshr (red) (L), medaka Lhr (blue) with tilapia Lhr (red) (P), and medaka alpha subunit (red) with tilapia alpha subunit (green) (S). The gonadotropin subunits and gonadotropin receptors are shown in ribbon representation. 3-D structures were developed using the I-Tasser online tool. Protein models were further prepared using Maestro tool in Schrödinger software. Structural alignment and superposition was performed using Schrödinger Maestro (Schrödinger Release, 2018-3) and verified by Pymol (PyMOL, Molecular Graphics System, version 2.0 Schrödinger).

reaching the late maturity stage.

The reason for the division of fish in distinct standard body length groups for the developmental *fshr* and *lhr* gene expression profile of medaka in the present study was based on the evidence that initiation of puberty is strongly correlated with body length in female zebrafish (Chen and Ge, 2013), which is well documented in mammals (Kennedy and Mitra, 1963). In zebrafish, the timing of female sexual maturation was not associated to age, but rather to body growth. In our previous study we used a similar classification and showed that the increase of *lhb* expression and the Fsh and Lh pituitary content are correlated to body length during the first sexual maturation of male medaka (Burow et al., 2019). A lower limit regarding body length, weight or age at which all fish were completely immature could not be determined, although the limit must be below 14 mm body length, 47 mg body weight, and 46 dpf. Important to note is that future studies should include even shorter specimens (< 14 mm) since fully mature specimens were found down to a body length of 15.5 mm.

Not much is known about the regulation of gonadotropin receptors in male fish. Studies in mammals have demonstrated that *FSHR* and *LHR* mRNA levels are differentially regulated by their cognate hormones, dependent on the stage of gonadal development (Ascoli et al., 2002; Walker and Cheng, 2005). *LHR* mRNA levels in Leydig cell progenitors in rats were significantly increased after the presence of LH and testosterone (Shan et al., 1995). In contrast, exposure of cultured immature porcine Leydig cells to recombinant LH resulted in down-regulation of *LHR* mRNA (Lejeune et al., 1998). FSH upregulated *FSHR* transcript levels in Sertoli cells in 1 to 10 day old rats, whereas in 20 day old rats, *FSHR* mRNA was downregulated and no effect was detected in adults (Maguire et al., 1997; Dahia and Rao, 2006). It is not known whether gonadotropin receptor genes in fish are controlled similarly, and whether other factors are involved (Maugars and Schmitz, 2008).

We further examined the intra- and interspecies ligand specificity of Japanese medaka and Nile tilapia Fshr and Lhr using a receptor-trans-activation assay. In both medaka and tilapia, the two gonadotropins could activate their own cognate receptor as expected (see Aizen et al. (2012b) for full examination of tilapia gonadotropins on their cognate receptors). Interestingly, mdFshβα was also able to activate the mdLhr, with similar potency. On the other hand, mdLhβα activated the mdFshr, but with lower potency than mdFshβα (14-fold higher concentration was needed). Generally, in contrast to mammals where FSHR and LHR are highly specific to their receptors, studies in teleosts suggest that the bioactivity of fish gonadotropins seems to be less specific, due to promiscuous hormone-receptor interactions. In some teleosts, Fshr and Lhr can be cross-activated by Lh and Fsh, respectively, and thus teleost receptor specificity is unclear (Levavi-Sivan et al., 2010). This was first demonstrated for coho salmon where Fshr did not discriminate between Fsh and Lh binding, whereas the Lhr bound only Lh (Miwa et al., 1994; Yan et al., 1992). From later studies demonstrating the pharmacological characterization of cloned receptors of African catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003; Vischer et al., 2004), channel catfish (Kumar et al., 2001a,b), zebrafish (Kwok et al., 2005; So et al., 2005), rainbow trout (Sambroni et al., 2007), Japanese eel (Kazeto et al., 2008), and Atlantic salmon (Andersson et al., 2009), it can be suggested that the larger but still limited functional selectivity of the teleost Fshr for both gonadotropins could depend on the fish species or taxon, while the Lhrs seem to be specific for their corresponding ligand, like their mammalian counterparts (Aizen et al., 2012b). This seems not the case in medaka, since we revealed that both mdFshβα and mdLhβα were able to activate the mdLhr. This suggests that both mdFshr and mdLhr are less discriminating to Fsh and Lh, respectively, compared to other species mentioned above. Surprisingly, the present study showed that tiLhr could be activated by both recombinant tiFshβα and tiLhβα. However, in a previous study, using both tiFshr and tiLhr, tiFshβα and tiLhβα were more selective to their cognate receptors (Aizen et al., 2012a). The reason why MPE did not activate the mdFshr in this study could be due to the pituitary extract containing too low amounts of Fsh to activate the receptors. Inter-species ligand promiscuity in this study revealed interesting findings, since tiFshβα and TPE could activate the mdLhr. Cross-species reactivity for the mdFshr was detected by activation with TPE only. MPE could activate the tiFshr and the tiLhr, indicating cross-reactivity also for these receptors. Cross-species reactivity was also shown by Aizen et al. (2012b), in that tiLhr was activated by human chorionic gonadotropin (hCG), and Japanese eel Lhr could be activated by human FSH, hCG and Manchurian trout (*Brachymystax lenok*) Fsh. Furthermore, human FSHR was activated by porcine FSH and bovine FSH.

We could not find a complete rationalization for the interspecies cross-activation of medaka Fshr and Lhr by TPE, or for the activation of tilapia Fshr and Lhr by MPE, which was shown in clear contrast to the specificity of recombinant gonadotropins. Since recombinant gonadotropins and natural gonadotropins from pituitary extracts have similar primary structures, we expected that they would show the same cross-activation activity. Furthermore, recombinant gonadotropins should fold into the same functional three-dimensional conformation as endogenous gonadotropins, since both recombinant gonadotropins were able to activate its cognate receptors. The interspecies cross-activation, showed by the pituitary extracts and the selectivity of the recombinant gonadotropins, should therefore depend on other factors. Perhaps a difference in N-linked oligosaccharide composition between pituitary gonadotropins and recombinant gonadotropins could be one candidate. *P. pastoris* can glycosylate recombinant proteins in the same glycosylation sites as mammalian expression systems. It has been previously used to generate biologically bioactive glycoproteins in mammals (e.g. Blanchard et al., 2008) and teleosts (e.g. Aizen et al., 2017), but the composition of the N-linked glycan could be different. Comparison of the gonadotropin β-subunit showed a difference in N-glycosylation site position between medaka Fshβ and Nile tilapia Fshβ that could explain the difference of activity between medaka and Nile tilapia Fshβα. A recent study analyzing the role of individual N-glycosylation sites, by N-glycosylation site mutagenesis using recombinant Japanese eel Lh in mammalian CHO cells, revealed that glycosylation of both α and β-subunits play a pivotal role in signal transduction of Japanese eel Lhr (Byambaragchaa et al., 2018), in a similar way it was shown for mammals (Bishop et al., 1994). Another possible explanation for the activation by pituitary extract is the presence of other hormones than gonadotropins; possibly Tsh can activate the gonadotropin receptors.

With regards to the biological meaning for gonadotropin receptor promiscuity in medaka, a recent study using Transcription Activator-Like Effector Nuclease (TALEN) reported interesting findings by disrupting gonadotropin receptor expression in zebrafish (Zhang et al., 2015a). Neither Fshr nor Lhr deficient zebrafish mimicked deficiencies of their ligands. The study suggests that this is possibly because

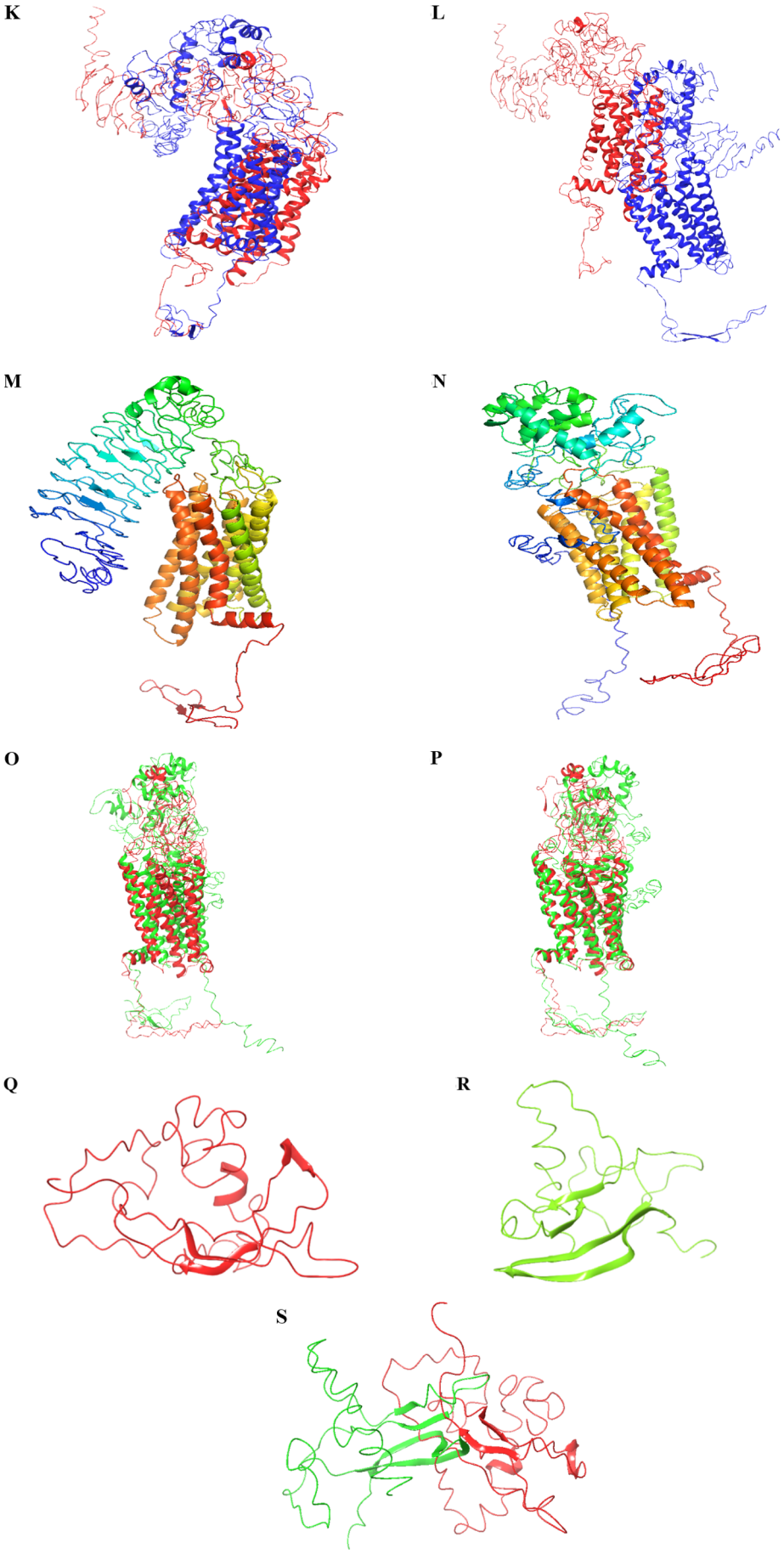

Fig. 8. (*continued*)

zebrafish Fshr can be activated by both Fsh and Lh proteins. In medaka, it was revealed that Fshr was indispensable to folliculogenesis, and the disruption of the *fshr* gene caused a complete failure of follicle activation, which was followed by masculinization of females into males (Murozumi et al., 2014). Compared to this, it was indicated that Lhr is not crucial for zebrafish reproduction in either sex (Zhang et al., 2015a). Furthermore, Fsh-deficient zebrafish (*fshb*$^{-/-}$) were fertile in both sexes, but development of both ovary and testis was significantly delayed; with an ensuing delay in female, but not male puberty onset. Compared to this, Lh-deficient zebrafish (*lhb*$^{-/-}$) showed normal gonadal growth, but females failed to spawn and were therefore infertile. Neither the *fshb* nor *lhb* mutation alone seemed to influence gonadal differentiation. Moreover, Fsh may play a role in maintaining female status, probably through regulation of ovarian aromatase (Zhang et al., 2015b; reviewed by Trudeau, 2018). In addition, Takahashi et al. (2016) reported that *fshb* and *lhb* deficiency using TALEN had no effect on male fertility in medaka, possibly due to overlapping actions of Fsh and Lh.

Furthermore, this study suggests that there are also regions in the receptors participating in recognition and/or activation, which could explain the activation profile. Amino acid sequence comparison of the extracellular domain, which is involved in the hormone recognition, between medaka and tilapia Fshr and Lhr, revealed some divergence. An additional N-glycosylation site was predicted, positioned between the cysteine residue 2 and 3 of the hinge region in Nile tilapia Fshr. In contrast, an additional N-glycosylation site was predicted between the cysteine residue 3 and 4 of the hinge region in medaka Lhr. The hinge region of the glycoprotein hormone receptors has been shown to be involved in hormone binding and induction of signaling pathway (Jiang et al., 2012; Kleinau et al., 2017). In addition, implication of the hinge region in receptor activation has been reported for TSH (Mueller et al., 2010). Furthermore, a previous study indicated that residues outside of the established hormone-receptor interface region may be involved in gonadotropin interactions with their receptors (Aizen et al., 2012b). To unravel possible causes for the divergence observed in the transactivation assay, an amino acid sequence alignment, as well as a protein structural super-position and structural alignment between medaka Fshβ and Lhβ ligands and Fsh and Lh receptors and those of other teleosts, including Nile tilapia, were performed.

Structural modeling is a powerful tool for analyzing and predicting ligand-receptor binding and activation (Niv et al., 2006; Brockhoff et al., 2010; Yarnitzky et al., 2010). The biological activity of a molecule is highly dependent on its structure. Structural superposition and alignments are key tools to understand the homology between two or more proteins, and to establish structural similarity and equivalence between them. These tools help to define the similarity of biological activity and affinity towards the similar binding regions on their receptors. In the present study, we used structural modeling and sequence analysis to reveal the molecular basis of cross-type hormone specificity and cross-species activity. Similar amino acid residues, which determine ligand selectivity, can be present in both gonadotropins and their receptors, thus explaining the observed promiscuity (Levavi-Sivan et al., 2010). Related to the findings presented in this study, this can be a reason for cross-reactivity. Various studies have determined important regions of gonadotropins and their receptors, which are crucial for binding selectivity. These are for instance the “seatbelt region” of hCG (Campbell et al., 1991) and specific LRRs and residues in human LHCGR and human FSHR (Bogerd et al., 2005; Bogerd, 2007). Studies investigating chimeric analogs of the human gonadotropin β subunits demonstrated that particularly the region between the 10th and 12th conserved cysteine residues, named the seatbelt region, is essentially involved in determining receptor specificity. Especially the net charge difference on the region between the 10th and 11th cysteine residue (named determinant loop) between LH (and hCG) on the one hand, and FSH (and TSH) on the other hand, were suggested to have separated LHR- from FSHR-/TSHR-activating properties (Campbell et al., 1997; Han et al., 1996). Additional sequence divergence within the C-terminal seatbelt region and outside the seatbelt segment were revealed as further separation of specific FSH, TSH and LH activities (Grossmann et al., 1997). Studying these regions in medaka and tilapia gonadotropins and their receptors, resulted in residues that might partially explain the detected activity profile. The seatbelt region for both Fshβ and Lhβ in both species was well aligned and superpositioned. The net charge for the determinant loop was determined to be 1 for medaka Fshβ, −1 for Nile tilapia Fshβ, −1 for medaka Lhβ, and −2 for Nile tilapia Lhβ. Since only medaka Fshβ shows a positive charge, this might be a reason for its slightly promiscuous nature that our study advocates. There might be factors present in the pituitary extracts that influence the net charge on this region or of the entire protein. The revealed high similarity in *trans*-membrane regions between Lhr of medaka and Nile tilapia, compared to the Fshr, could partially be explained by the replacement of the conserved cysteine residue by tyrosine at glycine 581 in the transmembrane domain of medaka Fshr that leads to impairment of the transmembrane domain conformation, without abolishing receptor function.

Taken together, we present the first characterization of the interactions between gonadotropins and gonadotropin receptors in Japanese medaka and Nile tilapia. Our results demonstrate that each gonadotropin activated its own cognate receptor but also complex relations and cross-species reactivity between the ligands and receptors. A tissue screen of medaka *fshr* and *lhr* expression revealed that both receptor genes are highly expressed in ovary and testis, and at low levels in various non-gonadal tissues. A developmental gene expression profile in testes of *fshr* and *lhr* suggests that both transcripts increase during pubertal development. We used ligand–receptor structural modeling from fish species representing two teleost orders to investigate the molecular basis of ligand selectivity. The biodiversity of hormones and receptors, and cross-species reactivity, provide insights to understand hormone specificity, which could reveal crucial determinants of selectivity.

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Appendix A. Supplementary data

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References

Aizen, J., Kasuto, H., Levavi-Sivan, B., 2007. Development of specific enzyme-linked immunosorbent assay for determining LH and FSH levels in tilapia, using recombinant gonadotropins. Gen. Comp. Endocrinol. 153, 323–332.

Aizen, J., Kobayashi, M., Selicharova, I., Sohn, Y.C., Yoshizaki, G., Levavi-Sivan, B., 2012a. Steroidogenic response of carp ovaries to piscine FSH and LH depends on the reproductive phase. Gen. Comp. Endocrinol. 178, 28–36.

Aizen, J., Kowalsman, N., Kobayashi, M., Hollander, L., Sohn, Y.C., Yoshizaki, G., Niv, M.Y., Levavi-Sivan, B., 2012b. Experimental and computational study of inter- and intra- species specificity of gonadotropins for various gonadotropin receptors. Mol. Cell. Endocrinol. 364, 89–100.

Aizen, J., Hollander-Cohen, L., Shpilman, M., Levavi-Sivan, B., 2017. Biologically active recombinant carp LH as a spawning-inducing agent for carp. J. Endocrinol. 232, 391–402.

Albertson, R.C., Kocher, T.D., 1999. Shape differences in the trophic apparatus of two Lake Malawi cichlid species and their hybrid progeny - a landmark-based morphometric approach. Am. Zool. 395, 115A-115A.

Al-Hader, A.A., Lei, Z.M., Rao, C.V., 1997. Neurons from fetal rat brains contain functional luteinizing hormone/chorionic gonadotropin receptors. Biol. Reprod. 56, 1071–1076.

Andersson, E., Nijenhuis, W., Male, R., Swanson, P., Bogerd, J., Taranger, G.L., Schulz,

R.W., 2009. Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (Salmo salar L.) ovaries. Gen. Comp. Endocrinol. 163, 329–339.
Ascoli, M., Fanelli, F., Segaloff, D.L., 2002. The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocr. Rev. 23, 141–174.
Baron, D., Houlgatte, R., Fostier, A., Guiguen, Y., 2005. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod. 73, 959–966.
Biran, J., Ben-Dor, S., Levavi-Sivan, B., 2008. Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. Biol. Reprod. 794, 776–786.
Bishop, L.A., Robertson, D.M., Cahir, N., Schofield, P.R., 1994. Specific roles for the asparagine-linked carbohydrate residues of recombinant human follicle stimulating hormone in receptor binding and signal transduction. Mol. Endocrinol. 8, 722–731.
Blanchard, V., Gadkari, R.A., George, A.V., Roy, S., Gerwig, G.J., Leeflang, B.R., Dighe, R.R., Boelens, R., Kamerling, J.P., 2008. High-level expression of biologically active glycoprotein hormones in Pichia pastoris strains-selection of strain GS115, and not X-33, for the production of biologically active N-glycosylated 15N-labeled phCG. Glycoconj. J. 25, 245–257.
Bogerd, J., 2007. Ligand-selective determinants in gonadotropin receptors. Mol. Cell. Endocrinol. 260–262, 144–152.
Bogerd, J., Blomenröhr, M., Andersson, E., van der Putten, H.H., Tensen, C.P., Vischer, H.F., Granneman, J.C., Janssen-Dommerholt, C., Goos, H.J., Schulz, R.W., 2001. Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (Clarias gariepinus). Biol. Reprod. 64, 1633–1643.
Bogerd, J., Granneman, J.C., Schulz, R.W., Vischer, H.F., 2005. Fish FSH receptors bind LH: how to make the human FSH receptor to be more fishy? Gen. Comp. Endocrinol. 142, 34–43.
Breton, B., Billard, R., Jalabert, B., 1973. Spécificité d'action et relations immunologiques des hormones gonadotropes de quelques téléostéens. Ann. Biol. Anim. Biochim. Biophys. 13, 347–362.
Brockhoff, A., Behrens, M., Niv, M.Y., Meyerhof, W., 2010. Structural requirements of bitter taste receptor activation. Proc. Natl. Acad. Sci. U.S.A. 107, 11110–11115.
Burow, S., Fontaine, R., von Krogh, K., Mayer, I., Nourizadeh-Lillabadi, R., Hollander-Cohen, L., Cohen, Y., Shpilman, M., Levavi-Sivan, B., Weltzien, F.-A., 2019. Medaka Follicle-stimulating hormone (Fsh) and Luteinizing hormone (Lh): Developmental profiles of pituitary protein and gene expression levels. Gen. Comp. Endocrinol. 272, 93–108.
Byambaragchaa, M., Kim, D.J., Kang, M.H., Min, K.S., 2018. Site specificity of eel luteinizing hormone N-linked oligosaccharides in signal transduction. Gen. Comp. Endocrinol. 268, 50–56.
Campbell, R.K., Dean-Emig, D.M., Moyle, W.R., 1991. Conversion of human choriogonadotropin into a follitropin by protein engineering. Proc. Natl. Acad. Sci. U.S.A. 88, 760–764.
Campbell, R.K., Bergert, E.R., Wang, Y., Morris, J.C., Moyle, W.R., 1997. Chimeric proteins can exceed the sum of their parts: implications for evolution and protein design. Nat. Biotechnol. 15, 439–443.
Chang, W.C., Lee, T.Y., Shien, D.M., Hsu, J.B., Horng, J.T., Hsu, P.C., Wang, T.Y., Huang, H.D., Pan, R.L., 2009. Incorporating support vector machine for identifying protein tyrosine sulfation sites. J. Comput. Chem. 30, 2526–2537.
Chauvigné, F., Tingaud-Sequeira, A., Agulleiro, M.J., Calusinska, M., Gómez, A., Finn, R.N., Cerdà, J., 2010. Functional and evolutionary analysis of flatfish gonadotropin receptors reveals cladal- and lineage-level divergence of the teleost glycoprotein receptor family. Biol. Reprod. 82, 1088–1102.
Chen, W., Ge, W., 2013. Gonad differentiation and puberty onset in the zebrafish: evidence for the dependence of puberty onset on body growth but not age in females. Mol. Reprod. Dev. 80, 384–392.
Dahia, C.L., Rao, A.J., 2006. Regulation of FSH receptor, PKI b, IL-6 and calcium mobilization: possible mediators of differential action of FSH. Mol. Cell. Endocrinol. 247, 73–81.
Dukic-Stefanovic, S., Walther, J., Wosch, S., Zimmermann, G., Wiedemann, P., Alexander, H., Claudepierre, T., 2012. Chorionic gonadotropin and its receptor are both expressed in Human retina, possible implications in normal and pathological conditions. PloS One 7, e52567.
Gether, U., 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr. Rev. 21, 90–113.
Grossmann, M., Szkudlinski, M.W., Wong, R., Dias, J.A., Ji, T.H., Weintraub, B.D., 1997. Substitution of the seat-belt region of the thyrotropin (TSH) b subunit with the corresponding regions of choriogonadotropin or follitropin confers luteotropic, but not follitropic activity to chimeric TSH. J. Biol. Chem. 272, 15532–15540.
Han, Y., Bernard, M.P., Moyle, W.R., 1996. hCGb residues 94–96 alter LH activity without appearing to make key receptor contacts. Mol. Cell. Endocrinol. 124, 151–161.
Hodne, K., von Krogh, K., Weltzien, F.-A., Sand, O., Haug, T.M., 2012. Optimized conditions for primary culture of pituitary cells from the Atlantic cod (Gadus morhua). The importance of osmolality, pCO(2), and pH. Gen. Comp. Endocrinol. 178, 206–215.
Hollander-Cohen., L, Golan, M., Aizen, J., Shpilman, M., Levavi-Sivan, B., 2018. Characterization of carp gonadotropins: Structure, annual profile, and carp and zebrafish pituitary topographic organization. Gen. Comp. Endocrinol. 264, 28–38.
Hu, L., Wada, K., Mores, N., Krsmanovic, L.Z., Catt, K.J., 2006. Essential role of G protein-gated inwardly rectifying potassium channels in gonadotropin-induced regulation of GnRH neuronal firing and pulsatile neurosecretion. J. Biol. Chem. 281, 25231–25240.
Jeng, S.R., Yueh, W.S., Chen, G.R., Lee, Y.H., Dufour, S., Chang, C.F., 2007. Differential expression and regulation of gonadotropins and their receptors in the Japanese eel, Anguilla japonica. Gen. Comp. Endocrinol. 154, 161–173.
Ji, T.H., Grossmann, M., Ji, I., 1998. G protein-coupled Receptors. I. Diversity of receptor-ligand interactions. J. Biol. Chem. 273, 17299–17302.
Jiang, X., Liu, H., Chen, X., Chen, P.H., Fischer, D., Sriraman, V., Yu, H.N., Arkinstall, S., He, X., 2012. Structure of follicle-stimulating hor- mone in complex with the entire ectodomain of its receptor. Proc. Natl. Acad. Sci. U.S.A. 109, 12491–12496.
Kasuto, H., Levavi-Sivan, B., 2005. Production of biologically active tethered tilapia LHβα by the methylotrophic yeast Pichia pastoris. Gen. Comp. Endocrinol. 140, 222–232.
Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi, S., Yamauchi, K., 2008. Japanese eel follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): production of biologically active recombinant Fsh and Lh by Drosophila S2 cells and their differential actions on the reproductive biology. Biol. Reprod. 79, 938–946.
Kazeto, Y., Kohara, M., Tosaka, R., Gen, K., Yokoyama, M., Miura, C., Miura, T., Adachi, S., Yamauchi, K., 2012. Molecular characterization and gene expression of Japanese eel (Anguilla japonica) gonadotropin receptors. Zoolog. Sci. 29, 204–211.
Kennedy, G.C., Mitra, J., 1963. Body weight and food intake as initiating factors for puberty in the rat. J. Physiol. 166, 408–418.
Kleinau, G., Worth, C.L., Kreuchwig, A., Biebermann, H., Marcinkowski, P., Scheerer, P., Krause, G., 2017. Structural-functional features of the thyrotropin receptor: A Class A G-protein-coupled receptor at work. Front. Endocrinol. 8, 86.
Kobayashi, T., Pakarinen, P., Torgersen, J., Huhtaniemi, I., Andersen, Ø., 2008. The gonadotropin receptors FSHR and LHR of Atlantic halibut (Hippoglossus hippoglossus) -2. Differential follicle expression and asynchronous oogenesis. Gen. Comp. Endocrinol. 156, 595–602.
Kumar, R.S., Ijiri, S., Trant, J.M., 2001a. Molecular biology of channel catfish gonadotropin receptors: 1. Cloning of a functional luteinizing hormone receptor and preovulatory induction of gene expression. Biol. Reprod. 64, 1010–1018.
Kumar, R.S., Ijiri, S., Trant, J.M., 2001b. Molecular biology of the channel catfish gonadotropin receptors: 2. Complementary DNA cloning, functional expression, and seasonal gene expression of the follicle-stimulating hormone receptor. Biol. Reprod. 65, 710–717.
Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol. 189, 541–554.
Kwok, H.F., So, W.-K., Wang, Y., Ge, W., 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors – evidence for their distinct functions in follicle development. Biol. Reprod. 72, 1370–1381.
Lei, Z.M., Rao, C.V., Kornyei, J.L., Licht, P., Hiatt, E.S., 1993. Novel expression of human chorionic gonadotropin/luteinizing hormone receptor gene in brain. Endocrinology 132, 2262–2270.
Lejeune, H., Sanchez, P., Chuzel, F., Langlois, D., Saez, J.M., 1998. Timecourse effects of human recombinant luteinizing hormone on porcine Leydig cell specific differentiated functions. Mol. Cell. Endocrinol. 144, 59–69.
Levavi-Sivan, B., Aizen, J., Avitan, A., 2005. Cloning, characterization and expression of the D_2 dopamine receptor from the tilapia pituitary. Mol. Cell. Endocrinol. 236, 17–30.
Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437.
Maguire, S.M., Tribley, W.A., Griswold, M.D., 1997. Follicle-stimulating hormone (FSH) regulates the expression of FSH receptor messenger ribonucleic acid in cultured Sertoli cells and in hypophysectomized rat testis. Biol. Reprod. 56, 1106–1111.
Maugars, G., Dufour, S., 2015. Demonstration of the Coexistence of duplicated Lh receptors in teleosts, and their origin in ancestral Actinopterygians. PLoS ONE 10, e0135184.
Maugars, G., Schmitz, M., 2006. Molecular cloning and characterization of FSH and LH receptors in Atlantic salmon (Salmo salar L.). Gen. Comp. Endocrinol. 149, 108–117.
Maugars, G., Schmitz, M., 2008. Expression of gonadotropin and gonadotropin receptor genes during early sexual maturation in male atlantic salmon parr. Mol. Reprod. Dev. 75, 403–413.
Miwa, S., Yan, L.G., Swanson, P., 1994. Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. Biol. Reprod. 50, 629–642.
Molés, G., Zanuy, S., Muñoz, I., Crespo, B., Martínez, I., Mañanós, E., Gómez, A., 2011. Receptor specificity and functional comparison of recombinant sea bass (Dicentrarchus labrax) gonadotropins (FSH and LH) produced in different host systems. Biol. Reprod. 84, 1171–1181.
Mueller, S., Jaeschke, H., Günther, R., Paschke, R., 2010. The hinge region: an important receptor component for GPHR function. Trends Endocrinol. Metab. 21, 111–122.
Murozumi, N., Nakashima, R., Hirai, T., Kamei, Y., Ishikawa-Fujiwara, T., Todo, T., Kitano, T., 2014. Loss of follicle-stimulating hormone receptor function causes masculinization and suppression of ovarian development in genetically female medaka. Endocrinology 155, 3136–3145.
Niv, M.Y., Skrabanek, L., Filizola, M., Weinstein, H., 2006. Modeling activated states of GPCRs: the rhodopsin template. J. Comput. Aided Mol. Des. 20, 437–448.
Nyuji, M., Kitano, H., Shimizu, A., Lee, J.M., Kusakabe, T., Yamaguchi, A., Matsuyama, M., 2013. Characterization, localization, and stage-dependent gene expression of gonadotropin receptors in chub mackerel (Scomber japonicus) ovarian follicles. Biol. Reprod. 88, 148.
Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H., Nagahama, Y., 1999a. Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (Oncorhynchus rhodurus). Biochem. Biophys. Res. Commun. 263, 584–590.
Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H., Nagahama, Y., 1999b. The duality of fish gonadotropin receptors: cloning and functional characterization of a

second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (Oncorhynchus rhodurus). Biochem. Biophys. Res. Commun. 265, 366–371.
Oba, Y., Hirai, T., Yoshiura, Y., Kobayashi, T., Nagahama, Y., 2001. Fish gonadotropin and thyrotropin receptors: the evolution of glycoprotein hormone receptors in vertebrates. Comp. Biochem. Physiol. B 129, 441–448.
Ogiwara, K., Fujimori, C., Rajapakse, S., Takahashi, T., 2013. Characterization of luteinizing hormone and luteinizing hormone receptor and their indispensable role in the ovulatory process of the medaka. PLoS ONE 8, e54482.
Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Ann. Rev. Biochem. 50, 465–495.
Rahman, M.A., Ohta, K., Yamaguchi, A., Chuda, H., Hirai, T., Matsuyama, M., 2003. Gonadotropins, gonadotropin receptors and their expression during sexual maturation in yellowtail, a carangid fish. Fish Physiol. Biochem. 28, 81–83.
Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: The European Molecular Biology Open Software Suite. Trends in Genetics 16, 276–277.
Rocha, A., Gómez, A., Galay-Burgos, M., Zanuy, S., Sweeney, G.E., Carrillo, M., 2007a. Molecular characterization and seasonal changes in gonadal expression of a thyrotropin receptor in the European sea bass. Gen. Comp. Endocrinol. 152, 89–101.
Rocha, A., Gómez, A., Zanuy, S., Cerdá-Reverter, J.M., Carrillo, M., 2007b. Molecular characterization of two sea bass gonadotropin receptors: cDNA cloning, expression analysis, and functional activity. Mol. Cell. Endocrinol. 272, 63–76.
Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., 2009. Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. Gen. Comp. Endocrinol. 162, 265–275.
Roy, A., Kucukural, A., Zhang, Y., 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nat. Protoc. 5, 725–738.
Sambroni, E., Le Gac, F., Breton, B., Lareyre, J.J., 2007. Functional specificity of the rainbow trout (Oncorhynchus mykiss) gonadotropin receptors as assayed in a mammalian cell line. J. Endocrinol. 195, 213–228.
Shan, L., Hardy, D.O., Catterall, J.F., Hardy, M.P., 1995. Effects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen receptors, and steroidogenic enzymes in rat Leydig cell progenitors in vivo. Endocrinology 136, 1686–1693.
So, W.K., Kwok, H.F., Ge, W., 2005. Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits–their spatial-temporal expression patterns and receptor specificity. Biol. Reprod. 72, 1382–1396.
Takahashi, A., Kanda, S., Abe, T., Oka, Y., 2016. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. Endocrinology 157, 3994–4002.
Trudeau, V.L., 2018. Facing the Challenges of Neuropeptide Gene Knockouts: Why Do They Not Inhibit Reproduction in Adult Teleost Fish? Front. Neurosci. 12, 302.
Vassart, G., Pardo, L., Costagliola, S., 2004. A molecular dissection of the glycoprotein hormone receptors. Trends Biochem. Sci. 29, 119–126.
Vischer, H.F., Bogerd, J., 2003. Cloning and functional characterization of a gonadal luteinizing hormone receptor complementary DNA from the African catfish (Clarias gariepinus). Biol. Reprod. 68, 262–271.
Vischer, H.F., Granneman, J.C., Linskens, M.H., Schulz, R.W., Bogerd, J., 2003. Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. J. Mol. Endocrinol. 31, 133–140.
Vischer, H.F., Marques, R.B., Granneman, J.C., Linskens, M.H., Schulz, R.W., Bogerd, J., 2004. Receptor-selective determinants in catfish gonadotropin seatbelt loops. Mol. Cell. Endocrinol. 224, 55–63.
Walker, W.H., Cheng, J., 2005. FSH and testosterone signaling in Sertoli cells. Reproduction 130, 15–28.
Weltzien, F.-A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). Comp. Biochem. Physiol. 137, 447–477.
Weltzien, F.-A., Pasqualini, C., Vernier, P., Dufour, S., 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. Gen. Comp. Endocrinol. 142, 134–142.
Yan, L., Swanson, P., Dickhoff, W.W., 1992. A two-receptor model for salmon gonadotropins (GTH I and GTH II). Biol. Reprod. 47, 418–427.
Yang, E.J., Nasipak, B.T., Kelley, D.B., 2007. Direct action of gonadotropin in brain integrates behavioral and reproductive functions. Proc. Natl. Acad. Sci. U.S.A. 104, 2477–2482.
Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., Zhang, Y., 2015. The I-TASSER Suite: Protein structure and function prediction. Nat. Methods 12, 7–8.
Yarnitzky, T., Levit, A., Niv, M.Y., 2010. Homology modeling of G-protein-coupled receptors with X-ray structures on the rise. Curr. Opin. Drug Discov. Devel. 13, 317–325.
Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225, 131–185.
You, S., Kim, H., Hsu, C.C., El Halawani, M.E., Foster, D.N., 2000. Three different turkey luteinizing hormone receptor (tLH-R) isoforms I: characterization of alternatively spliced tLH-R isoforms and their regulated expression in diverse tissues. Biol. Reprod. 62, 108–116.
Zhang, Y., 2008. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 9, 40.
Zhang, Z., Zhu, B., Ge, W., 2015b. Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. Mol. Endocrinol. 29, 76–98.
Zhang, Z., Lau, S.W., Zhang, L., Ge, W., 2015a. Disruption of zebrafish follicle-stimulating hormone receptor (fshr) but not luteinizing hormone receptor (lhcgr) gene by TALEN leads to failed Follicle activation in females followed by sexual reversal to males. Endocrinology 156, 3747–3762.