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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 mdFshβa was able to activate the mdLhr, and mdLhBa 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, tiFshβa 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 tiFsh $\beta\alpha$, tiLh $\beta\alpha$, and TPE, as well as for tiFshr and tiLhr due to stimulation by mdFshβa, mdLhβa, 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 tworeceptor 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 CaCl₂, 0.33 mM MgSO₄, 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

 oPCR primers used in the present study.

Target	Reference	Primer sequence	Accession number	Amplicon size (nt)	Efficiency
lhr	This study	Forward: 5'-CAGTCTGAGGGGGGATTGAAA-3'	ENSORLP00000013664	110	2.04
		Reverse: 5'-AGGAGACGGATGTGAAATCG-3'			
fshr	This study	Forward: 5'-ATCAGTGGCGATGCCTTCGTGG-3'	ENSORLP0000039837	79	2.00
		Reverse: 5'-CGGGCAAAGAGCTGATGGCTGT-3'			
18s	Burow et al., 2019	Forward: 5'-CCTGCGGCTTAATTTGACTC-3'	AB105163.1	118	2.02
		Reverse: 5'-AACTAAGAACGGCCATGCAC-3'			
rpl7	Burow et al., 2019	Forward: 5'-TGCTTTGGTGGAGAAAGCTC-3'	NM_001104870	98	2.03
-		Reverse: 5'-TGGCAGGCTTGAAGTTCTTT-3'			
gapdh	This study	Forward: 5'-CCTCCATCTTTGATGCTGGT-3'	XM_004077972.3	75	2.01
		Reverse: 5'- ACGGTTGCTGTAGCCAAACT-3'	_		

480 SYBR Green I Master (Roche). All samples were run in duplicate, each composed of 5 μ l mastermix, 5 μ M of each of forward and reverse primer, and 3 μ l of 10x diluted cDNA sample in a total volume of 10 μ l. 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-13acetate (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 $Lh\beta\alpha/Fsh\beta\alpha$ and 500–0.12 ng/ml for tilapia Lh $\beta\alpha$ /Fsh $\beta\alpha$). 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 Fsh $\beta\alpha$ (mdFsh $\beta\alpha$), medaka Lh $\beta\alpha$ (mdLh $\beta\alpha$), Nile tilapia Fsh $\beta\alpha$ (tiFsh $\beta\alpha$), Nile tilapia Lh $\beta\alpha$ (tiLh $\beta\alpha$), 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% CO₂ 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), pcmdFshr, 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 mdLhßa, mdFshßa, tiLhßa, tiFshßa (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 ligandreceptor 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, a-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 \pm 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 (posthoc 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).

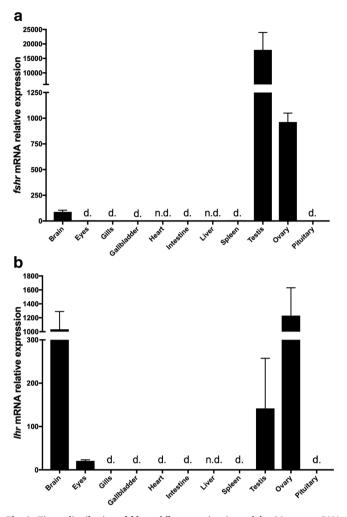


Fig. 1. Tissue distribution of *fshr* and *lhr* transcripts in medaka. Messenger RNA levels for *fshr* (A) and *lhr* (B) were assayed by qPCR in various tissues of adult medaka: brain, eyes, gills, gallbladder, heart, intestine, liver, spleen, testis, ovary, and pituitary (n = 3). d., detected but not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

Concerning the mdLh $\beta\alpha$, 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 mdLh $\beta\alpha$ (EC50 = 0.47 ± 0.05 ng/ml) (Table 2, Fig. 3D) and mdFsh $\beta\alpha$ (EC50 = 1.38 ± 0.09 ng/ml) (Table 2, Fig. 3D) in a dose-dependent manner. In addition, mdFsh $\beta\alpha$ 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 mdLh $\beta\alpha$, however to a lower extent than mdFsh $\beta\alpha$, (EC50 = 6.44 ± 0.11 ng/ml) (Table 2, Fig. 3C).

Tilapia gonadotropins, tiFsh $\beta\alpha$ and tiLh $\beta\alpha$, 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 tiFsh $\beta\alpha$ and tiLh $\beta\alpha$ could activate mdLhr, but to a much lower extent and with lower EC50s compared to mdFshr (EC50 = 1.3 x $10^{-5} \pm 0.09$ 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

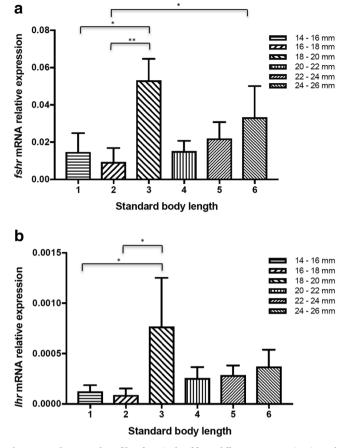


Fig. 2. Developmental profile of testicular *fshr* and *lhr* gene expression in male medaka. Transcript levels for *fshr* (A) and *lhr* (B) were measured in medaka classified according to standard body length (group 1 14–16 mm, group 2 16–18 mm, group 3 18–20 mm, group 4 20–22 mm, group 5 22–24 mm, group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

Table 2

Effective half-maximum concentration (EC₅₀, 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 \pm SEM (n = 3).

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

(Fig. 5F).

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 Fsh β and Lh β 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 Fsh β (59% identity, 71.8% similarity) than the medaka Lh β and tilapia Lh β (66.7% identity, 80.3% similarity). Based on alignment of cysteine residues, medaka and tilapia Lh β conserved the twelve cysteines common to glycoprotein hormone β -subunits, while Fsh β of medaka, contrary to most other Acanthopterygii, conserved the position of only 11 cysteine residues

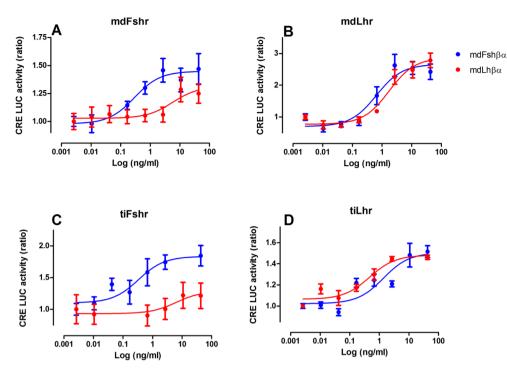
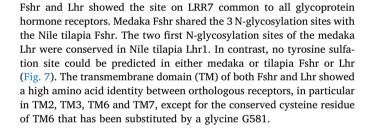


Fig. 3. Receptor-transactivation assay of medaka and Nile tilapia gonadotropin receptors for characterization of intra- and interspecies ligand selectivity using homologous and heterologous ligands. Medaka Fsh $\beta\alpha$ and Lh $\beta\alpha$ induced transcriptional activity in COS-7 cells that were 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 Fshßa and Lhßa (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 Fsh β , the third cysteine is shifted three residues downstream, whereas it has been lost in both tilapia and European seabass Fsh β . The latter two instead have an additional cysteine upstream of cysteine C1. Medaka and the other acanthopterygian Fsh β show shorter length compared to Atlantic salmon Fsh β , European eel (*Anguilla anguilla*) Fsh β , and human FSH β , including several amino acid deletions between C1 and C2, C5 and C7, and C7 and C8. For both medaka Fsh β and Lh β , a single N-glycosylation site is present, positioned between C1 and C2 for medaka Fsh β , and between C10 and the C11 for medaka Lh β (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).

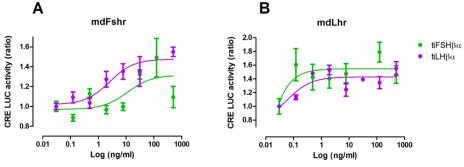
The ECD in medaka Lhr consisted of 11 successive conserved LRRs, as in other vertebrate Lhrs, while an additional LRR to the 11 LRRs was found positioned between the LRR2 and LRR3 in medaka Fshr. This extra LRR was also observed in Nile tilapia and European seabass Fshr. Three and four N-glycosylation sites were predicted for medaka Fshr and Lhr, respectively. Among these N-glycosylation sites, both medaka



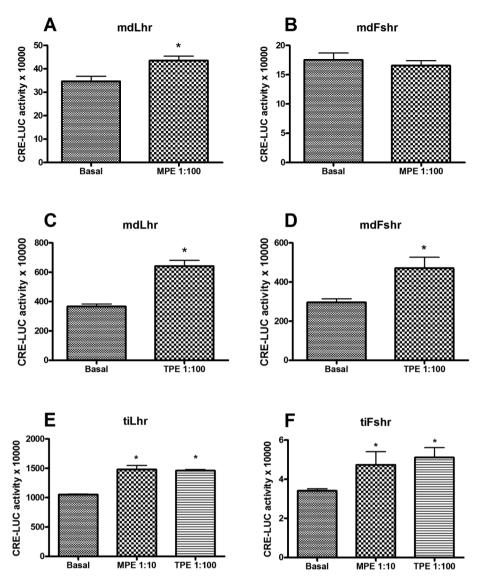
3.6. Structural models of gonadotropin β -subunits, α -subunits and receptors

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 Fsh β (Fig. 8A) and Nile tilapia Fsh β (Fig. 8B), as well as medaka Lh β (Fig. 8E) and Nile tilapia Lh β (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 cotransfected 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.



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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, $p \le 0.05$.

be highly conserved between medaka Fsh β (Fig. 8A) and Nile tilapia Fsh β (Fig. 8B), as well as between medaka Lh β (Fig. 8E) and Nile tilapia Lh β (Fig. 8F). Superposition of both gonadotropin β -subunits revealed structural homology between the Fsh β of both medaka and Nile tilapia (Fig. 8C), and between Lh β of medaka and Nile tilapia (Fig. 8G). Structure alignment between medaka and Nile tilapia Fsh β (Fig. 8D),

and between $Lh\beta$ of medaka and Nile tilapia (Fig. 8H) detected high
similarity in β sheet domains. The $\alpha\mbox{-subunit}$ 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 FSHß	MKTLQFFFL	FCCWKAICCNSCELT <mark>NIT</mark> IA	IEKEECRFCISI <mark>N</mark>	TTWCAGYCYTRDLVYKDP	60
Eel Fshß	MHLAVTALCLTLA	<i>PILARA</i> STSCGLA <mark>NIS</mark> IS	VENEECGGCVTF <mark>N</mark>	TTACAGLCFTODSVYKSS	62
Salmon Fshß	MYCTRLRTLQLVVMATLWVT	PVRAGTDCRYGCRLN <mark>NMT</mark> IT	VEREDCHGSIT	ITTCAGLCETTDLNYOST	69
Tilapia Fshß	MQLVVMAAVLA	<i>LAGA</i> EQDCSSGCRPK <mark>NIS</mark> L-	-PVDTCGFVD	TTICEGQCFQKDPNFIHT	57
Medaka Fshß	MQLVVMAAALV	LAEVGQVSSFSCHPK <mark>NVS</mark> I-	-PVESCGISGCVH	TTICEGRCYHEDPNYISY	60
Seabass Fshß	MOLVVMVAVLA	LARAGOGCSFGCHPTNISI-	-OVESCGLTEVIY	TTICEGOCYHEDLVYLSH	60
Human LHß	MEMLQGLLLLLLSMGG	AWASREPLRPWCHPINAILA	VEKEGCPVCITVN	TTICAGYCPTMMRVLQAV	68
Eel Lhβ	MSVYPECTWLLFVCLCHL				69
Salmon Lhß	MLGLHVGT-LISLFLCIL	<i>LEPVEG</i> SLMOPCOPI <mark>NOT</mark> VS	LEKEGCPTCLVIO	TPICSGHCVTKEPVFKSP	68
Tilapia Lhß	MMAQISRMLLALMLSLFVGASTFI	LSPAAAFOLPPCOLI <mark>NOT</mark> VS	LEKEGCPSCHPVE	TTICSGHCITKDPVIKIP	75
Medaka Lhß	MISRVSRVMFLLMLSFILGTSTFLWS.	LAPAAAFOLPYCOPVKOKLS	LOKEGCSGCHTVE	TTVCSGHCLTKDPLMKI-	76
Seabass Lhß	MAVQASRVMFPLVLSLFLGATSSIWP	LATAEAFOLPPCOLINOTVS	LEKEGCPKCHPVE	TTICSGHCITKDPVIKIP	77
'	~	~ ~ ~			
	6 7	8 9 10	11	12	
Human FSHß	6 ARPKIQKT <mark>C</mark> TFKELVYETVRVPG <mark>C</mark> AH				9
Human FSHβ Eel Fshβ		HADSLYTYPVATQCHCGKCD	SDSTDCTVRGLGP	SYCSFGEMKE 12	
	ARPKIQKTCTFKELVYETVRVPGCAH	HADSLYTYPVATQCHCGKCD GMDLHFTYPVALSCECSKCN	SDST <mark>DC</mark> TVRGLGP TDST <mark>DC</mark> GPLNTEV	SYCSFGEMKE 12 SGCLTH 12	7
Eel Fshβ	ARPKIQKTCTFKELVYETVRVPGCAH LKPYPQQACNFRDVVYETVHLPGCPS	HADSLYTYPVATQCHCGKCD SMDLHFTYPVALSCECSKCN GVDP-FFIPVAKSCDCIKCK	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13	7 7
Eel Fshβ Salmon Fshβ	ARPKIQKTCTFKELVYETVRVPGCAHI LKPYPQQACNFRDVVYETVHLPGCPS WLPRSQGACNFKDWSYEKVYLEGCPS	HADSLYTYPVATQCHCGKCD GMDLHFTYPVALSCECSKCN GVDP-FFIPVAKSCDCIKCK GFIYPVARKCECTACN	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13 PSC 11	7 7 3
Eel Fshβ Salmon Fshβ Tilapia Fshβ	ARPKIQKTCTFKELVYETVRVPGCAHI LKPYPQQACNFRDVVYETVHLPGCPS WLPRSQGACNFKDWSYEKVYLEGCPS DDWPKQKTCN-GEWSYEVKYTEQCPR	HADSLYTYPVATQCHCGKCD SMDLHFTYPVALSCECSKCN GVDP-FFIPVAKSCDCIKCK GFIYPVARKCECTACN GFKYPVAKSCECTTCN	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI TRTTYCGRLSADM	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13 PSC 11 PSC 11	7 7 3 7
Eel Fshβ Salmon Fshβ Tilapia Fshβ <mark>Medaka Fshβ</mark>	ARPKIQKTCTFKELVYETVRVPGCAHI LKPYPQQACNFRDVVYETVHLPGCPSX WLPRSQGACNFKDWSYEKVYLEGCPSX DDWPKQKTCN-GEWSYEVKYTEQCPR EDHPKEKICS-GDWSYEVKFIEGCPV0	HADSLYTYPVATQCHCGKCD SMDLHFTYPVALSCECSKCN GVDP-FFIPVAKSCDCIKCK GFIYPVARKCECTACN GFKYPVARSCECTTCN GVTYPVARNCECTTCN	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI TRTTYCGRLSADM TENTDCGRFPGDI	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13 PSC 11 PSC 11 PSCLSF 12 PSCLSF 12	7 7 3 7 0
Eel Fshβ Salmon Fshβ Tilapia Fshβ Medaka Fshβ Seabass Fshβ	ARKIQKTCTFKELVYETVRVGCAH LKPYPQQACNFRDVYETVHLPGCPS: WLPRSQGACNFKDWSYEKVYLEGCPS: DDWFKQKTCN-GEWSYEVKYTEQCPR: EDHPKEKICS-GDWSYEVKFIEGCPV: YERPEQRICN-GDWSYEVKHIKGCPV:	HADSLYTYPVATQCHCGKCD MMLHFTYPVALSCECSKC SVDP-FFIPVASSCDCIKCK 3FIYPVARKCECTACN 3FKYPVARKCECTTCN GVDPVVSFPVALSCRCGPCR	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI TRTTYCGRLSADM TENTDCGRFPGDI RSTSDCGGPKDHP	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13 PSC 11 PSCLSF 11 PSCLSF 12 LTCDHPQLSGLLFL 14	7 7 3 7 0 1
Eel Fshβ Salmon Fshβ Tilapia Fshβ Medaka Fshβ Seabass Fshβ Human LHβ	ARPKIQKTCTFKELVYETVRVPGCAH LKPYPQQACNFKDVYETVHLPGCPS: WLPRSQCACNFKDWYEKVYLEQCPS: DDWPKQKTCN-GEWSYEVVTEQCPR EDHPKEKICS-GDWSYEVKFIEGCPV YERPEQRICN-GDWSYEVKFIEGCPV LPPLPQVCTYRDVRFESIRLPGCPR	HADSLYTYPVATQCHCGKCD SMDLHFTYPVALSCECSKCN SUDP-FFIPVAKSCDCIKCK GFIVPVAKSCECTACN GFKYPVAKSCECTTCN GVTYPVARNCECTTCN SVDPVVSFVALSCCCNLCT	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI TRTTYCGRLSADM TENTDCGRFPGDI RSTSDCGGPKDHP MDTSDCAIQSLRP	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 12 PSC 11 PSC	7 7 3 7 0 1 0
Eel Fshβ Salmon Fshβ Tilapia Fshβ Medaka Fshβ Seabass Fshβ Human LHβ Eel Lhβ	ARKIQKTCTFKELVYETVRVPGCAH LKPYPQQACNFRDVYETVHLPGCPS WLPRSQGACNFKDWSYEKVYLEGCPS DDWFKQKTCN-GEWSYEVKTTEQCPW EDHFKEICS-GDWSYEVKTTEQCPV YERPEQRICN-GDWSYEVKHIKGCPV LPPLPQVVCTYRDVRFSIRLPGCPK LSTVYQRVCTYRDVRFSIRLPGCPK	HADSLITYPVATQCHCGKCD SMDLHFTYPVALSCCSKCM SUDP-FFIVVARSCDCIKCK 3FIVPVARKCECTACN 3FKYPVARKCECTTCN GVDPVVSFPVALSCCCTTCN GVDPVVSFPVALSCDCNLCT WVDPHVTPVALSCDCSLCN	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI TRTTYCGRLSADM TENTDCGREPGDI RSTSDCGGPKDHP MDTSDCAIQSLRP MDTSDCTIESLQP	SYCSFGEMKE 12 SGCLTH	7 7 3 7 0 1 0 2
Eel Fshß Salmon Fshß Tilapia Fshß Medaka Fshß Seabass Fshß Human LHß Eel Lhß Salmon Lhß	ARPKIQKTCTFKELVYETVRVPGCAH LKPYPQQACNFKDWYETVRLPGCPS: WLPRSQGACNFKDWSYEKVYLEGCPS: DDWFKQKTCN-GEWSYEVKTFEQCPW EDHPKEKICS-GDWSYEVKHIKGCPV YERPEQRICN-GDWSYEVKHIKGCPV LPPLPQVVCTYRDVRFESIRLPGCPW LSTVYQRVCTYRDVRFESIRLPGCPW FSTVYQHVCTYRDVRYETVRLPCRPU FSTVYQHVCTYRDVRYETIRLPDCPP	HADSLYTYPVATQCHCGKCD SMDLHFYPVALSCECSKCN SVDP-FFIPVAKSCCIKCK 3FIYPVAKSCECTICN 3FVVVAKSCECTTCN 3VTYPVAKSCECTTCN 3VDPVVSPVALSCAGPCR SVDPVVSPVALSCCSLCN WDPHVTYPVALSCCSLCN WDPHVTYPVALSCCGRCA	SDSTDCTVRGLGP TDSTDCCPLNTEV TDNTDCDRISMAT A-NTDCGTLSGYI TRTTYCGRLSADM TENTDCGRFPGDI RSTSDCGGPKDHP MDTSDCAIQSLRP MDTSDCTIESLQP MDTSDCTIESLQP	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13 PSC 11 PSCSF 11 PSCLSF 12 LTCDHPOLSGLIFL 14 DFCMSQRAS 1-PA DFCITHRALMOGNUM 14 DFCMNDTFFY2 14	7 7 3 7 0 1 0 2 5
Eel Fshβ Salmon Fshβ Tilapia Fshβ Medaka Fshβ Seabass Fshβ Human LHβ Eel Lhβ Salmon Lhβ Tilapia Lhβ	ARFKIQKTCTFKELVYETVRVPGCAH LKPYPQQACNFRDVYETVHLPGCPS WLPRSQGACNFKDWSYEKVYLEGCPS DDWFKQKTCN-GEWSYEVKTTEQCPK EDHFKEKICS-GDWSYEVKTTEQCPV YERPEQRICN-GDWSYEVKHTKGCPV LPPLQVVCTYRDVFESIRLPGCPR LSTVYQRVCTYRDVRYETVRLPDCPP FSTVYQHVCTYRDVRYETVRLPDCPP FSNVQHVCTYRDVRYETFELPDCPP	HADSLYTYPVATCCHCGKCD SMDLHFYPVALSCCSKCD SVDP-FFIPVARSCCIKCK GFIYPVARSCCTTCN GFYVPVARSCCTTCN GVTYPVARSCCTTCN SVDPVVFPVALSCCGCD SVDPHVTPVALSCCORSLON SVDPIVTPVALSCCICGRCA SVDPIVTPVALSCHCGACI	SDSTDCTVRGLGP TDSTDCGPLNTEV TDNTDCDRISMAT A-NTDCCTLSGYI TRTTYCGRLSADM TENTDCGRFPGDI RSTSDCGGPKDHP MDTSDCAIQSLRP MDTSDCTIESLQP MMTSDCTFESMQP	SYCSFGEMKE 12 SGCLTH 12 PSCLGSPLEM 13 PSC 11 PSCLSF 12 LTCDHPQLSGLLFL 14 DFCMSQRAS_L=PA 14 DFCITHRALMDGNMW 14 DFCWNDIPFYY 14 DFCWNDIPFYY 14	7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7

Fsh β and Lh β with other teleost representatives as well as with the human FSHB and LHB 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).

Fig. 6. Amino acid sequence alignment of medaka

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Human FSHR	LRR1 > LRR1 > S2 S2 S2 S2 S2 S2 S2 S2 S2 S2 S2 S2 S2
Eel Fshr Salmon Fshr	MTPLWVLFLGLVSGTSCV-AMHVCLA <mark>NGTTRSFFCV</mark> GSRVNQMPAVIPKNTSYIE 54 MMKMKKIMKMLLCVLGCVCVSQAEVAMVNSGTTFTYLCMG-NT-ITHMPTHIPKNTTNLE 58
Tilapia Fshr	MMLVMTLMMLLIVTIKMAAASAHGSEMDIRPG-FHPSLAKQ <mark>TSCLSY</mark> QVMFGVTAFPS <mark>NIS</mark> -NAQCLE 66
<mark>Medaka Fshr</mark> Seabass Fshr	<mark>LDDVCF</mark> QVELGFSTFLRSISS <mark>NSTVVN</mark> 52 MVVMILIMLMILVIKTATASVPGPEMDVKPGVETSLAKR <mark>TLSFCY</mark> QLKFGVTEIPSSISS <mark>NTTCLE</mark> 66
Human LHCGR	MKQRFSALQLLKLLLLQPPLPRALREALCPE-PCNCVPDGALRCPGPTAGLTRLS 55
Eel Lhrl Salmon Lhrl	MSNLLLWTMWRLVMLSTVLQVRSGWTFSCP-VICQCTE-QSFRCTRETQLNSSARSSLINNIR 61 MAPARQTVWVLLALSGVLNLRSCWTFTCP-SICKCSLKSDTCYKDTGEKLSCTRETQLKSQAAPSFIRNLR 70
Tilapia Lhr1	MALREVWLLFALSGVLNARSCCAYTCP-AICRCTA-DSFQCSKETQLASRTGPTSVLRLR 58
<mark>Medaka Lhrl</mark> Seabass Lhrl	MAPRAVWLLVALSGVLNARSCQAYPCH-PICRCTP-DTFQCNRDTQLAAGTAEHRLR 50 MAPRAVWLLVALSGVLNVRSCWAYTCP-AICRCTA-DTFQCSRDTQLASRAAAASVPRLW 56
Eel Lhr2 Salmon Lhr2	MDLRLSVLFLLFPSVMCMWGFMTISDFQCPG-VCMCSP-KTIRCNRVTELSLPVAQRDSYRRLT 62 MMSISLLFLFYPSVLLFFGFGCRYASSFVCPG-ICRCSS-NTIRCNNITEKSVPMSERGPRLV 61
Tilapia Lhr2	MWTSPSVSLLLFVSFFHGCRNFVCPR-ICRCFS-NAIRCNNITQGSAPVMDHRDRRLF 56
Seabass Lhr2	FS- <mark>NTIRCNNV</mark> TQGSALMMDHRD <mark>KRLF</mark> 59
Human FSHR	LRR2
Eel Fshr	FVUTRLEVIOKGAFSGFGDLEKTEISONDVLEVIEADVFSNLPKHEIRIEKA 105 FKITQLRLIPRAAFSEHELSRIMVSENGALEGIAAHAFSN SNLVEITITKS 107 FKQTHIRVEPREAFINLL
Salmon Fshr Tilapia Fshr	FKQTHIRVFPREAFTNLLQLTAIVLTENGMLESIGAFAFANLPRLTEITITKS 111 VKQTQIREIQQGTLSSLQHLMELTISENDLLESIGAFAFSGLPHLTKILISKNAALRNIGAFVFSNLPELSEIIITKS 144
Medaka Fshr	IKQTQITVIDQSIFTGLWHLEKLTILDNDKLLSICPSAFANLPRLFDVSIXQNMALKKIGAFAFSNLPALTEIEITKS 130
Seabass Fshr Human LHCGR	VKQTEIVVIPQGALNSLQHLRKLTIWENDKLESINEFAFASLSQLTDIFISGNVALKNIGAFAFSDLPELTEITITKS 144 LAYLPVKVIPSOAFRGLN
Eel Lhrl	LTHLPLQEVPSNTFRDLHNVDRIEISQSDSIRIFRARAFHSLHSLQEVLIHNM 114
Salmon Lhrl Tilapia Lhrl	LTHLPLTEVPSNAFRGLINVSRIEISQSDCTRYIRNHAFLSLHSLTEIQIQNI 123 LTHLPLKRVPSHAFKELINITIIEISQSDCITHIQTHAFLSLYSLAQISVQNI 111
Medaka Lhrl	LTHLPLKQVPTHAFKELM
Seabass Lhr1 Eel Lhr2	LTHLEFACEVPTHAFKELI
Salmon Lhr2	LKHLTMSTIASHTFDGLRRVQHIEIGQSVALETIETLAFNNLLDLNEIFIKNT 114
Tilapia Lhr2 Seabass Lhr2	VKQTEIVVIPQAALMSLQHLAKLITIMENDKLESINEFAFASLSQITDIFISGNVALKNIGAFAFSDLPELTEITITKS 144 LAYLPVKVIPSQAFKGLM
	► LRR5 ► LRR6 ► LRR7
Human FSHR Eel Fshr	NNLLYINPEAFQNLPNLQYLL SNTGIKHLPDVHKIHSLQKVLLOIQDNINIHTIERNSFVGLSFESV-ILW N 178 KNLVFIEKGAFWNLSRLKYLT SNTGLKSLPDFSKINSAAKDFLFDLQDNVNMKVIHPNAFLGLSSETIRELR T 182
Salmon Fshr	KHLVVIHHQAFMGLPKLSHLTICNTGLRVLPNFSRIHSTALTFLLDLQDNVHIVIIPSNAFLGLTTNTIDELRLT 186
Tilapia Fshr <mark>Medaka Fshr</mark>	KHLSFIHPDAFRNMARLRFLT SNTGLRIFPDFSKIHSTACFLLDLQDNSHIKRVPANAFRGLCTQTFAEIR T 218 RHLTSIHPDAFRSLVGLRRLI TNTGLRIFPDLSKIHSAVHRFMFDLQDNIHIQAIPANAFRGLCTQTVEEIR T 205
Seabass Fshr	KHLTHINPDAFKDIVKLKYLT ANTGLRLFPDFTKIHSTGLLLFDLHDNSHIERVPANAFKGLCTQTIPEIRLT 218
Human LHCGR Eel Lhrl	KNLRYIEPGAFINLPRLKYLS CNTGIRKFPDVTKVFSSESNFIL ICDNLHITTIPGNAFQGMNNESVT-LK Y 182 KNLEFIKKGAFSDLPKLKYLS CNTGIREFPDLSTISSLEPLFFL VGDNIKIDTIPPNAFLGMTEEET-YMN V 188
Salmon Lhrl	RNLISIEKGAFTDLPRLRYLSICNTGIIHFPDFSTISSLVILE-YFFLELGDNIQLHSIPANAFQGITQESV-YMKII 199
Tilapia Lhr1 <mark>Medaka Lhr1</mark>	NSLRFIEKGAFADLPKLEYLS SNTGIAHFPDFTTISSLSPNIILMADMEIDIIPANSFQGITEEYVD-MN V 185 NSLRGIERGAFTDLPRLEFLT SNTGMMHFPDFTSVSSLTPSILLMLDMRIDVIPANSFRGMTKGHAN-MN V 182
Seabass Lhrl	NSLRVIEKGAFTDLPKLEYLSISNTGVIHFPDFTTISSLAPNIILFIADNMRIDIIPANSFQGITEEYVD-MNLV 185
Eel Lhr2 Salmon Lhr2	RRLAHISRKAFNNLPKLQYLSISNTGISLFPDLSAISSLQSNFILDICDNLNLQSIPSNAFIGMTKEHTD-MN_Y 189 RSLVHIARRTFNNLPKLRYLSISNTGITVFPDMTSIHSLEPWNQNFVLDICDNLYLLSIPVNAFVGMTTEYTA-MN_F 191
Tilapia Lhr2 Seabass Lhr2	RSIMHIDRGAFNNLPKL <mark>RYLSIS</mark> NTGIAVFPDVTSVSSLESEFVLDICDNIFLLEIPTNAFTGMSKEYV <mark>T-MN Y</mark> 183 RSIMHISRRTFNNLPKL <mark>HYLSIS</mark> NTGITLFPDITYINSLESEFILDICDNLYLLEIPPNAFIGLTKEYV <mark>T-MN Y</mark> 186
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Human FSHR	► LRR8 ► LRR9 ► LRR10 KNGIQEIHNCAF <mark>NGIQLDELNLSD</mark> NNNLEELPNDVFHGASGP <mark>VILDISR</mark> TRIHSLPSYGLENLKKLRARSTYNLKKLP 256
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Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Seabass Lhrl Seabass Lhrl Seabass Lhrl Salmon Lhr2 Tilapia Lhr2 Seabass Lhr2 Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR	KNGISEVESHAFNOK KIHKI FLMGNLQLSHMHNNSFKGAGGFGF DISRTALSSLFESVLGEVEHLSAVSVFSLFALP 264 RNGISEVESHAFNOK KIHKI FLMGNLQLSHMHNNSFKGAGGFGF DISRTALSSLFESVLGEVEHLSAVSVFSLFALP 266 RNGIREVASDAFNOK KMHRISLKGNKQLTHISGDAFVGSSELVV DISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFNOK KMHRISLKGNKQLTHINPNAFVGSSELVV DISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFNOK KMHRISLKGNKQLTHINPNAFVGSSELVV DISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFNOK KMHRISLKGNKQLTHINPNAFVGSSELVV DISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGFKEVGSHAFNOK KMHRISLKGNKQLTHINPNAFVGSSELVV DISETALGALPSVLGEVGLKILAESAYNLKELP 286 GNGFEEVQSHAFNOK KISSLIKKEN VLKGNRNLRKIHDEAFEGAGGFVI DISSTKLGALPSVLGEVUTUTARSAQGLKTLP 266 KNGFKEVIKSHAFNOK KLNTVIKGNNVLKNINQEDAFEGAGGFVI DVSSTALSSLPRGLENVLVLIARSTFYLKTLP 277 RNGFKEVIKSHAFNOK KLNTVIKDNUNKLNNIQEDAFEGAGGFSFI DVSSTALSSLPRGLENVLVLIARSTFYLKTLP 277 RNGFKEVIKSHAFNOK KLNTVIKDNNVLKNINGEDAFEGAGGFSFI DVSSTALSSLPRGLEAVLVLVRAKGAKLKSLP 263 RNGFKEVIQSHAFNOK KLNTVIKNNLKVIKHKALKHISDAFKGAFGFST DVSSTALSSLPFKGLEAVLVLVRAKGAVALKSLP 267 NNGIREVIDVAFNOK KLNTVIKNNLKVIKHKAFKGAVGFSFI DVSSTALSSLPFKGLEAVLVLVRAKGAVALKSLP 267 NNGIREVIDVAFNOK KLNTVIKNNLKVIHKDAFTGAFGFSFI DVSSTALSSLPFKGLEAVLVLVRAKGAVALKSLP 267 NNGIREVIDVAFNOK KLNTVIKNNLKVIHKDAFTGAFGFSFI DVSSTALSLPFKGLEAVLVLVRAKGAVALKSLP 267 NNGIREVIDVAFNOK KLNVIKNNLKVIHKDAFTGAFGFSFI DVSSTALSLPFKGLEAVLVLVRAKGAVALKSLP 267 NNGIREVIDVAFNOK KLNVIKNNLKVIHKDAFTGAFGFSFI DVSSTALSLPFKGLEAVLVLVRAKGAVALKSLP 267 NNGIREVIDVAFNOK KLNVIKNNLKVIHKDAFTGAFGFSFI DVSSTALSLPFKGLEAVLVLVRAKGAVALKSLP 264 MNGIREVIDVAFNOK KLNVIKNNLKVIHKDAFTGAFGFSFI DVSSTALSLPFKGLEAVLVLVRAKGAVGKS 312 NLDLFTOLFEANLT SHCCAFANSKKNMSVHELCDKPNIKQE
Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Medaka Lhrl Seabass Lhrl Eel Lhr2 Salmon Lhr2 Tilapia Lhr2 Seabass Lhr2 Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl	KNGISEVESHAFNOR KIHKI FLMGNLQLSHMHNNSFKGAGGFGF DISGTALSSLFESVLGEVEHLSAVSVFSLFALP 264 RNGISEVVESHAFNOR KMHR FLGGNQLTHISTAN-VGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFNOR KMHR FLGGNQLTHISTAN-VGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFNOR KMHR FLGGNKQLTHINPNA-VGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFNOR KMHR FLGGNKQLTHINPNA-VGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFNOR KMHR FLGGNKQLTHINPNA-VGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 286 GNGFEEVQSHAFNOR INSI ELKSNNKLEVHLEMMINGAFRGATGFKTLDISSTKLGALPSYGLESIGVLINTSSYLKKHP 260 GNGFEKUSGHAFNOR KHRILLS LLKSNNLIKNINPLANFGASGAFGFTLDISSTKLGALPSYGLESIGVLINTSSYLKKHP 260 RNGFKEIKSHAFNOR KLNNLIKNINFLNINGEDAFEGASGFTVLDVSSTALSALPAKGITRVOTIKATATFALKSLP 263 RNGFKEIQSHAFNOR KLNNLILDDNRFLSYIAEDAFEGATGFSFLDVSSTALSSLPPKGLEAVLVLIARSFYLKTP 277 RNGFKEIQSHAFNOR KLNNLIKNNKLKNHISDAFEGATGFSFLDVSSTALSSLPPKGLEAVLVLVARASAFALKTLP 263 RNGFKEIQSHAFNOR KLNNLIKNNKLKNHISDAFKGAFGFSFLDVSSTALSSLPPKGLEAVLVLVARASAFALKTLP 263 RNGFKEIQSHAFNOR KLNNLIKNNKLKNHISDAFKGAFGFSFLDVSSTALSSLPPKGLEAVLVLVARASAFALKTLP 264 NNGIREIHDYAFDOR KLNNLIKNNKLKNHINDAFKGAFGFSTLDVSSTALSSLPPKGLEAVLVLVARASAFALKTLP 264 NNGIREIHDYAFDOR KLNNLIKNNNLRVIHRDAFKGAFGFSTLDVSSTALSLPPKGLEAVLVLVARASAFALKSLP 264 NNGIRKIEBAHDOR KLNNLKNIKNNLRVIHRDAFGATGFGVLDVSSTALSLPPKGLEAVLVLVARASAFALKSLP 264 NNGIRKIEBALDY SEICCAFANWRRQISELHPICDKPNIKQEEVDYM-TQARGQRS 312 NLDLFTQLEAALLOF SEICCAFANWRRQISELHPICDKPNIKQE
Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Medaka Lhrl Seabass Lhrl Seabass Lhrl Salmon Lhr2 Tilapia Lhr2 Seabass Lhr2 Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhr1 Tilapia Lhr1	KNGISEVESHAFIGE KIHKI FLMGNLQLSHMHNNSFKGAGGFGE DISBTALSSLFPSVLGEVEHLSAVSVFSLFALP 264 RNGISEVVSADAFIGE KMHRISLKGNKQLTHISUARVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGE KMHRISLKGNKQLTHISGDAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGE KMHRISLKGNKQLTHINENAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGE KMHRISLKGNKQLTHINENAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGE KMHRISLKGNKQLTHINENAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGFKEVQSHAFNGT KISSLEVKULKGNNNLKKIHDEAFEGARGFTLDISSTKLGALPSYGLESIGULATSSVLKKLP 260 GNGFEEVQSHAFNGTKLKNILKGNNNLKNIHNENFEGARGFTLDISSTKLGALPSYGLESIGULATSSVLKKLP 260 RNGFKEIGSHAFNGTKLKNILKGNNNLKNIHNENTERSTGEFSTLDISSTALSLPPNGLRHVKFLKASHAYALKSLP 263 RNGFKEIGSHAFNGTKLKNILLRDNRFLSVIADAFEGARGFSFLVSSTALSSLPPKGLRQVTFLKASSAFALKSLP 260 RNGFKEIGSHAFNGTKLKNILLRDNRFLSVIADAFEGARGFSFLVSSTALSSLPFKGLRQVTFLKASSAFALKSLP 260 RNGFKEIGDYAFYGTKIKNILKNINKLKNIHSDAFKGAGFGSTLVSSTALSSLPFKGLRQVTLKASSAFALKSLP 260 RNGFKEIGDYAFYGTKIKNILKNINKLKVIHKAFKGAVGFSLVSSTALSSLPFKGLRQVTLKASSAFALKSLP 260 NNGIRKIHEHAFVGTKIKNILKNINKLKVIHKDAFTGATGFGVVVSATALTKLPSQGLESVLVLYARASAFCHKRP 269 NNGIRKIHEHAFVGTKIKNILKNINKLKVIHKDAFTGATGFGVVVSATALTKLPSQGLESVLVLFALSAFALKSLP 261 NNGIRRIHDYAFVGTKIKNILKNINKLKVIHKAFTGATGFGVVVSATALTKLPSQGLESVLVLFALSAFALKSLP 261 NNGIRRIHDYAFVGTKIKNIKNIKNINKLRVIHKAFTGATGFGVVVSATALTKLPSQGLESVLVLFALSAFATLKSLP 261 NNGIRRIHDYAFVGTKIKNIKNIKNIKNIKNIKSA PLELFTKLRQANLTP SHCCAFANNKRNMNSVHELCNFNGAQDN
Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Seabass Lhrl Seabass Lhrl Salmon Lhr2 Tilapia Lhr2 Salmon Lhr2 Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhr1 Tilapia Lhr1 Medaka Lhr1 Seabass Lhr1	KNGISEVESHAFIGE KIHKI FLMGNLQLSHMHENSEYGAGGFGE DISBTALSSLFESVLGEVEHLSAVSVFSLFALP 264 RNGISEVESHAFIGE KMHR.FLGGNQLTHISPNAVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGE KMHR.FLGGNQLTHISPNAVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGE KMHR.SLKGNKQLTHINPNAVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGE KMHR.SLKGNKQLTHINPNAVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGE KMHR.FLGGNKQLTHINPNAVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 286 GNGFEEVOGHAFNGE KMSK.VLKGNRNLRKIHDEAFEGAGFTLDISSTKLGALPSVGLSIGVUTUTARSAQGLKTLP 286 KNGFKEIKSHAFIGE KLHKLIKKDNKVLKNIHDEAFEGAGFTLDISSTKLGALPSVGLSIGVUTUTARSAQGLKTLP 266 RNGFKEIKSHAFIGE KLHNIILLDNRVLSHINGEDAFEGAGFTLDISSTALSALPAKGLRVVGHLKATATFALKSLP 263 RNGFKEIQSHAFNGE KLNNIILLDNRVLSHIQEDAFEGAGFFTLDISSTALSALPAKGLRVVGHLKATATFALKSLP 263 RNGFKEIQSHAFNGE KLNNIILLDNRVLSHIQEDAFEGAFGFSFLDVSSTALSSLPFKGLRQVRFLKASSAFALKTLP 263 RNGFKEIQSHAFNGE KLNNIILKDNRVLSHIGENAFEGATGFSFLDVSSTALSSLPFKGLRAVKLVLARATSVLKSLP 264 NNGIREIDOYAFNGE KLNNIILKNNRLKVIHKBAFKGAVGFSFLDVSSTALSSLPFKGLRAVKLVLVARGAYALKSLP 267 NNGIREIDOYAFNGE KLNVILKNNRLKVIHKBAFKGAVGFSTLDVSSTALSLPFKGLRAVLVLVARGAYALKSLP 267 NNGIREIDOYAFNGE KLNVILKNNRLKVIHKBAFKGAVGFSTLDVSSTALSLPFKGLRAVLVLVARGAYALKSLP 267 NNGIREIDOYAFNGE KLNVILKNNRLKVIHKDAFTGATGFGVUVSATALTKLPSQGLESVLVLFALSAATLKSLP 261 NNGIREIDOYAFNGE KLNVILKNNRLKVIHKDAFTGATGFGVUVSATALTKLPSQGLESVLVLFALSAYTLKSLP 261 NNGIREIDOYAFNGE KLNVILKNNRLKVIHRAFEGATGFGSUUVSATALTKLPPQGLESVLVLFAQSAYALKSLP 264 MNGIREIDAFGK KLDVILKNNRLKVIHRAFGSAFGFSTLDVSSTALFLFSGDON
Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Medaka Lhrl Seabass Lhrl Eel Lhr2 Salmon Lhr2 Tilapia Lhr2 Seabass Lhr2 Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhr1 Salmon Lhr1 Tilapia Lhr1 Medaka Lhr1	KNGISEVESHAFIOR KIHKI FLMGNLQLSHMHNNSFKGAGGPGF DISRTALSSLFPSVLGEVEHLSAVSVFSLFALP 264 RNGISEVVASDAFIOR KMHR FLGGNRQLTHISNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGR KMHR JELGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGR KMHR JELGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGR KMHR JELGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGR KMHR JELGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 286 RNGFREVQSHAFNGT KINS LIKGNKNLTHDEAFEGARGFTLDISSTKLAALPSQLLATSSVELKKLP 260 GNGFEVVQSHAFNGT KLNSLKKNLKNINVLENNIHDEAFEGARGFSLDVSSTALHSLPALGQQVTVUTARSAQGLKTLP 266 KNGFKEIGSHAFNGT KLNSLIKNVLKNNNLKVINNIENEGATGFSLDVSSTALSLPPNGLEHLKKLIARSTFVLKTLP 277 RNGFKEIKSHAFNGT KLNSLILRDNRFLSVIADDAFEGATGFSLVSSTALSSLPPKGLRQVTVUTARTATTALKSLP 260 RNGFKEIQSHAFNGT KLNSLIKNVLKNNKLKNHHSDAFEGATGFSLVSSTALSSLPPKGLRQVTFLKSSAFALKSLP 260 NNGFKEVQDHAFNGT KINKULKNNKLKVHHSDAFEGATGFSLVSSTALSSLPFKGLRQVTVLXARATALKSLP 260 NNGFKEVQDHAFNGT KINKULKNNRLRVIHRAFEKGAVGFSLVSSTALSLPFKGLRQVTVLXARATAGLKREP 269 NNGIRKIHEHAFNGT KINKULKNNRLRVIHRAFEKGAVGFGVUSSATAVEALFERGLAVLVLVARGAVALKSLP 261 NNGIRKIHEHAFNGT KINKULKNNRRLRVIHRAFEGATGFGVUSSATALFLPSQGLESVLVLFALSAFALKSLP 261 NNGIRKIHEMAFNGT KINKULKNNRRLRVIHRAFEGATGFGVUSSATALTKLPSQGLESVLVLFALSAYTLKSLP 261 NNGIRKIHEMAFNGT SHCCAFANNKRNSVHELCDKPNIKQE
Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Seabass Lhrl Seabass Lhrl Seabass Lhrl Seabass Lhrl Seabass Lhrl Seabass Lhrl Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Medaka Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Medaka Lhrl Seabass Lhrl	KNGISEVESHAFIGE KIHKI FLMGNLQLSHMHNNSFKGAGGFGE DISBTALSSLFPSVLGEVEHLSAVSVFSLFALP 264 RNGISEVVESHAFIGE KMHRISLKGNKQLTHISUARVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGE KMHRISLKGNKQLTHISGDAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGE KMHRISLKGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 283 RNGIREVASDAFIGE KMHRISLKGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 284 RNGIREVASDAFIGE KMHRISLKGNKQLTHINPNAFVGSSELVVLDISETAISSLPDNIISGLKKLLAESAYHLKILP 286 GNGFEEVQGHAFNGIKILSISLKKNPLCANNERVENDERVGNENGENENGENENGENENGENENGENENGENENGEN
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(caption on next page)

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 Fshß (supplementary data Fig. S1a), medaka and Nile tilapia Lhß (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 Fsh β (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 Lh β (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. Ihr 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

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	► TM2 ► TM3
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Human FSHR Eel Fshr Salmon Fshr Tilapia Fshr Seabass Fshr Human LHCGR Eel Lhrl Salmon Lhrl Tilapia Lhrl Medaka Lhrl	695 660 658 KQEC 701 KQEC 687 KPEF 701 YTEC 699 PK 704 GGAT 714 TT 698

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

Medaka Lhrl Seabass Lhrl Eel Lhr2 Salmon Lhr2 Tilapia Lhr2 Seabass Lhr2

--AT-- 688 --TT-- 695 ----- 700 GDLGTI 728 698 RELT-- 721

> 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

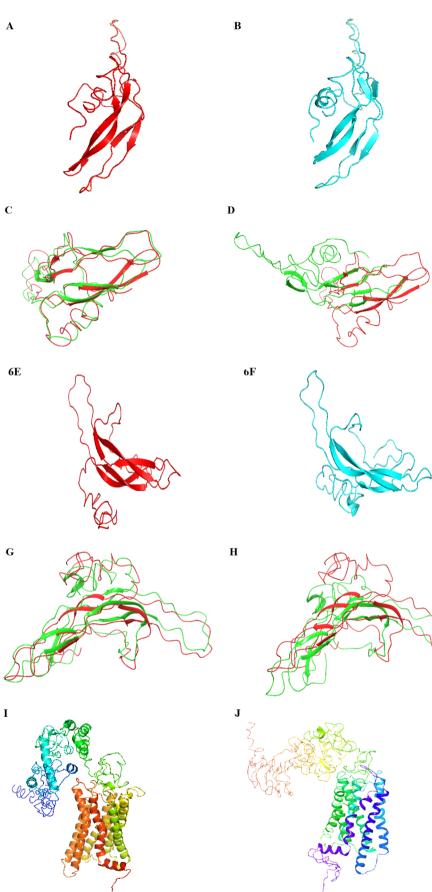


Fig. 8. Medaka and Nile tilapia gonadotropin β -subunit, α -subunit and gonadotropin receptor models. Structural three-dimensional model of medaka Fsh β (A), tilapia Fsh β (B), medaka Lh β (E), tilapia Lh β (F), medaka Fshr (I), tilapia Fshr (J), medaka Lhr (M), tilapia Lhr (N), medaka alpha subunit (Q), and tilapia alpha subunit (R). Superposition of medaka Fsh β (red) with tilapia Fsh β (green) (C), medaka Lh β (red) with tilapia Lh β (green) (G), medaka Fsh γ (red) (K), and medaka Lhr (blue) with tilapia Lhr (red) (O). Structure Alignment of medaka Fsh β (red) with tilapia Fsh β (green) (D), medaka Lh β (red) with tilapia Lh β (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 downregulation 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-transactivation 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, mdFsh $\beta\alpha$ was also able to activate the mdLhr, with similar potency. On the other hand, mdLh $\beta\alpha$ activated the mdFshr, but with lower potency than mdFshBa (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 mdFshBa and mdLhBa 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 tiFshBa and tiLhBa. However, in a previous study, using both tiFshr and tiLhr, tiFsh $\beta\alpha$ and tiLhBa 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 tiFshBa 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 crossactivation 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 Fshß and Nile tilapia Fshß that could explain the difference of activity between medaka and Nile tilapia Fsh $\beta\alpha$. A recent study analyzing the role of individual N-glycosylation sites, by Nglycosylation 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



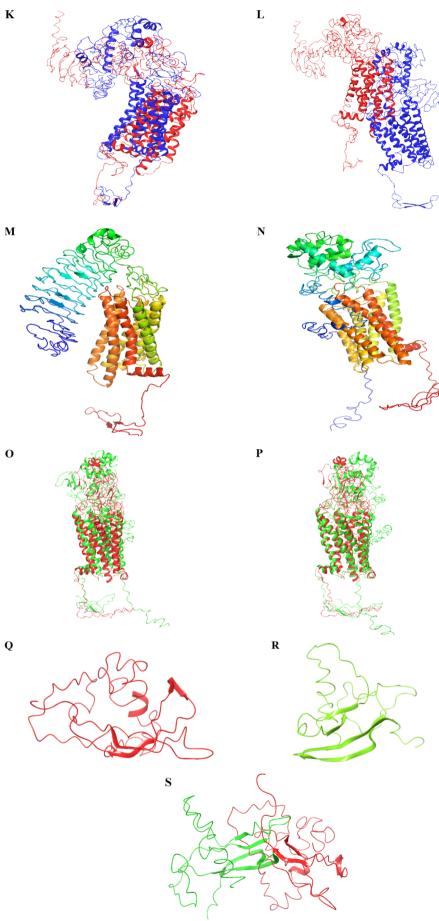


Fig. 8. (continued)

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 Fshß and Lhß 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 Fshß and Lhß in both species was well aligned and superpositioned. The net charge for the determinant loop was determined to be 1 for medaka Fsh β , -1 for Nile tilapia Fsh β , -1 for medaka Lh β , and -2 for Nile tilapia Lh β . Since only medaka Fsh β 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113276.

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