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Ontogeny of the specificity of gonadotropin receptors and gene expression in carp

Lian Hollander-Cohen, Benjamin Böhm, Krist Hausken and **Berta Levavi-Sivan**

Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food, and Environment, Hebrew University of Jerusalem, Rehovot, Israel

Correspondence should be addressed to B Levavi-Sivan: berta.sivan@mail.huji.ac.il

Abstract

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are the principle endocrine drivers of reproductive processes in the gonads of jawed vertebrates. Canonically, FSH recruits and maintains selected ovarian follicles for maturation and LH induces the stages of germinal vesicle breakdown and ovulation. In mammals, LH and FSH specifically activate cognate G-protein-coupled receptors that affect the proteins involved in steroidogenesis, protein hormone synthesis, and gametogenesis. This dual-gonadotropin model also exists in some fish species, but not in all. In fact, due to their diverse number of species, extended number of ecological niches, and remarkably flexible reproductive strategies, fish are appropriate as models to understand the co-evolution of gonadotropins and their receptors. In this study, we cloned and characterized the expression profile over the final stages of ovarian maturation of carp (*Cyprinus carpio*) LHCGR and FSHR. Expression of both gonadotropin receptors increased in the later stage of early vitellogenesis, suggesting that both LH and FSH play a role in the development of mature follicles. We additionally tested the activation of cLHCGR and cFSHR using homologous and heterologous recombinant gonadotropins in order to gain insight into an evolutionary model of permissive gonadotropin receptor function. These data suggest that carp (*Cyprinus carpio*) gonad development and maturation depends on a specific gonadotropin profile that does not reflect the temporally distinct dual-gonadotropin model observed in salmonids or mammals, and that permissive gonadotropin receptor activation is a specific feature of *Ostariophysi*, not all teleosts.

Key Words

- luteinizing hormone (LH)
- follicle-stimulating hormone (FSH)
- estradiol
- *Ostariophysi*
- GPCR
- dihydroxyprogesterone (17α, 20β, dihydroxy-4-pregnen, 3-one; DHP)

Endocrine Connections (2019) **8**, 1433–1446

Introduction

The brain–pituitary–gonad axis is a signaling cascade of hormones and their receptors that regulates reproduction in all jawed vertebrates. A milieu of stimulatory and inhibitory neuropeptides throughout different nuclei of the brain culminate in a signal to the pituitary gland to release the gonadotropins (GTHs). The pituitary GTHs include follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and together are responsible for gametogenesis, steroidogenesis, protein hormone synthesis, and ovulation throughout the time-sensitive process of gonad maturation. Generally speaking, circulating FSH levels are higher during gonad development and maturation, and circulating LH levels rise during the final stages of maturation and ovulation (1).

GTHs are heterodimeric proteins belonging to the cysteine-knot family, each consisting of a common α-subunit and a functionally specific β-subunit that provides binding specificity to their cognate receptors (2). FSH and LH exert their actions through the activation of specific G protein-coupled receptors (GPCRs) in the gonads. Glycoprotein hormone receptors (GpHRs) constitute subfamily A of the GPCRs and include the LH receptor (LHCGR), FSH receptor (FSHR), and thyroid-stimulating hormone receptor (TSHR). The GpHRs are characterized

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by a large N-terminal extracellular domain (ECD) that contains several leucine-rich repeat motifs (LRRs) and a low complexity 'hinge region' responsible for the selective and high-affinity binding of cognate hormones (3). The GpHRs canonically contain a seven transmembrane domain that undergoes a conformational change upon hormone binding to the ECD, revealing binding sites in the intracellular domain (ID) for G-protein interactions and arrestin-dependent receptor desensitization (1).

Ostariophysi is the second-largest superorder of teleost fish and includes Siluriformes (catfishes) and Cyprinidae (carps). This group represents 8000 species, 68% of freshwater species and 28% of known global fish species, and can be found on all major continents. The common carp is considered to be one of the most important aquaculture species globally (4), which is why we sought to characterize and study the gonadotropin receptors of this fish. We first cloned the cFSHR and LHCGR and studied their expression profile throughout the annual reproductive cycle. We then aimed to study the specific role of each of the carp gonadotropins at the final stages of oocyte maturation. We further studied the promiscuity of the cloned carp GTHRs by stimulation with homologous and heterologous recombinant fish gonadotropins in an attempt to build an evolutionary model of gonadotropin specificity. The accumulated results are interpreted to identify if different piscine reproductive strategies are driven by or a result of permissive gonadotropin actions.

Materials and methods

Cloning of cFSHR and cLHCGR and phylogenetic analysis

Carp total RNA extraction, synthesis of first cDNA strand, PCR procedure and cloning into a pGEMT Easy Vector were performed according to Hollander *et al.* (9) and Biran *et al.* (11). First- and second-strand cDNA were synthesized by means of 3′ and 5′ RACE PCR protocol with the SMART RACE cDNA Amplification kit (Clontech). The complete sequence of the receptor cDNAs were confirmed by sequence analysis of separate clones. To obtain the full length, we used start and stop primers for each receptor (Table 1).

Complete cDNAs were translated into the respective proteins and aligned to sequences of other organisms (accession numbers detailed in Supplementary Table 1, see section on supplementary data given at the end of this article) using the MUSCLE method in the MEGA7 program (Molecular Evolutionary Genetics Analysis, software version 7.0.26). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (5). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.0661)). The rate variation model allowed

Table 1 Primers used for cloning carp gonadotropin receptors.

Primer	Position	Sequence 5'–3'	Use
cFSHr 419R	446	GTCCAGAACGATGGGGCCTTCAGCTCC	PCR/5′ RACE cloning
cFSHr 443F	731	CACACCAGATGCATTCAACC	PCR cloning
cFSHr 276R	860	GCGGCTTGAAAAGAGCACTAGGAGG	PCR/5′ RACE cloning
cFSHr 191R	800	GAACCAGATTAGAACCCGCAGGAATG	PCR/5′ RACE cloning
cFSHr 426F	508	GGCCACCTCTGTCTACTCTC	PCR cloning
cFSHr start	1	ATGGTCTTG TTGATGATGC TG	Full cloning
cFSHr stop	1984	TCAGTACACTTGGGTGATGTG	Full cloning
cLHCGr 653F	653	GAACAACA AAGAGCCGCG TGTGATTC	PCR cloning
cLHCGr 304R	723	CATCCAGCATAGTGGGGCCAAACGC	PCR/5′ RACE cloning
cLHCGr 370F	420	CTGAGCATCTCCAACACTGG	PCR cloning
cLHCGr 387R	806	CAGAGTGAACGCTGAACGAGCCACCA	PCR/5′ RACE cloning
cLHCGr 1713R	1713	GGAAAGCCCCGACGTTGAACAGC	PCR/5′ RACE cloning
cLHCGr start2	1	TGA AGACAATACG GGACA ATGCTGAG	Full cloning
cLHCGr stop	2145	CTA CAC CCT TTG CAT CTG TGG	Full cloning
UPM		CTAATACGACTCACTATAGGGGC	5′ RACE cloning
NUPM		AAGCAAGTTGGTATCAACGCAGAGT	5′ RACE cloning
3′-RACE CDS		AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN	3′ RACE cloning
5′-RACE CDS		$(T)_{25}$VN	5′ RACE cloning

The position represents the location of the primer in the final sequence.

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for some sites to be evolutionarily invariable ([+*I*], 6.52% sites). The bootstrap consensus tree was inferred from 1000 replicates. The analysis involved 55 amino acid sequences. Evolutionary analyses were conducted in MEGA7 (6).

Structural modeling of cFSHR and cLHCGR

Three-dimensional models of cLHCGR and cFSHR were plotted using the I-TASSER server (7, 8) with the default I-TASSER parameters. Each receptor was divided into two domains; extracellular domain (nucleotides 1–345 for cFSHR and 1–380 for cLHCGR) and transmembrane domain which included the cytoplasmic domain (nucleotides 346–666 for cFSHR and 381–715 for cLHCGR). Domain prediction was performed using Phobius (http://phobius.sbc.su.se/). Visualization and super positioning of the models were performed using Swiss-PDB Viewer (http://spdbv.vital-it.ch/). The position of each extracellular domain in relation to its transmembrane was decided arbitrarily.

Fish

For the *in vivo* studies, common carp from the same long-term experiment previously published (9) were used. In short, fish were reared in earthen fishponds in Kibbutz Dan (Upper Galilee, Israel; 31°30′N, 34°45′E), under standard Israeli aquaculture (10). Eight to ten female fish were sampled monthly from September 2012 until August 2013. Their body weight (BW) and gonad weight (GW) were measured for gonadosomatic index (GSI) calculations, blood was withdrawn from the caudal vessels, and pituitary and gonads were collected into RNA Later (Life Technologies). Pituitaries were also collected into 100% ethanol for protein extraction and gonadal fragments were fixed in Bouin's fluid for histology (11). Pubertal stage of the fish gonads was determined according to histology (9, 12, 13, 14). Four stages were identified: I, Primary growth (PG)+ previtellogenic (PV); II, early vitellogenic (EV); III, mid-vitellogenic (MV); and IV, mature follicles (MFs).

For *in vitro* studies, fish ($n=8$, BW = 1.6 ± 0.3 kg) were kept indoor in a recirculating system in 500 L tanks at 24°C and a photoperiod of 14-h light. All experimental procedures complied with and were approved by the Animal Care and Use Guidelines of the Hebrew University and were also approved by the local administrative panel on laboratory animal care (AG-19-15969).

RNA extraction and real-time PCR

Total RNA was extracted from the gonads using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and integrity and quality of the purified RNA samples were evaluated by gel electrophoresis and nanodrop, respectively. cDNA was prepared as described previously (15). To assess the relative expression of *clhcgr* and *cfshr* mRNAs, mRNA levels were normalized against the geometric mean of two endogenous reference genes, *EF1α* and *β-actin*, using the comparative threshold cycle method (−ΔCT). The mRNA expression levels of both EF1α and β-actin were stable among all stages of oogenesis in the carp (Supplementary Fig. 1), as previously reported for tilapia (16) and Coho salmon (17). The RT quantitative PCR procedure was as described previously (11, 15). Six serial dilutions (1:2) were prepared from a gonad cDNA pool ($n=5$ fish), and the efficiencies of the specific gene amplifications were calculated by plotting Ct versus log template. Amplification was carried out in a LightCycler® 96 System (Roche), according to the manufacturer's protocol. All qPCR products were purified and sequenced in order to confirm that the correct targets were amplified. Validation of the RT-PCR: the dissociation curve analysis, absence of genomic DNA and the reverse-transcriptase negative control, were conducted as described previously (15). To improve presentation of results, the mean value of the primary growth (PG) stage was set to 1, so all normalized data are divided by the mean of this stage.

Tissue distribution of carp LHCGR and FSHR

Tissue samples were collected from three male and three female carp at different reproduction stages. Two micrograms of total RNA were extracted from each of the following tissues: the caudal brain, front brain, mid-brain, pituitary gland, spleen, gills, kidneys, muscles, fat, ovaries/testis, retina, heart, caudal and front intestines, and liver. cDNA samples were prepared as described previously (11). The tissue expression patterns of *clhcgr* and *cfshr* in the various tissues were analyzed by RT-PCR with the primer sets described in Table 1.

COS-7 reporter assays

Transient transfection, cell procedures, and stimulation protocols were generally performed as described previously (11, 18, 19). Briefly, COS-7 cells were grown in DMEM until confluent. Co-transfection of the receptors (3 µg/plate for cFSHr and 0.3 µg/plate for cLH) and a cAMP-response element-luciferase (CRE-Luc) reporter plasmid (at 3 µg/plate) was carried out with TransIT-X2® System (Mirus). The cells were serum-starved for 16 h, stimulated with various stimulants for 6 h, and then harvested and

analyzed as described previously (18). Experiments were repeated a minimum of three times from independent transfections and each was performed in triplicate.

Recombinant gonadotropins used in this study

Recombinant LH and FSH from tilapia (20, 21), sturgeon (*Acipenser gueldenstaedtii*) (22), and carp (9) were produced as biologically active, single-chain polypeptides in the methylotrophic yeast, *Pichia pastoris*. Recombinant tFSH and tLH were able to stimulate the release of 11-ketotestosterone (11-KT) from the testes, or the release of estradiol (E2) from the ovaries of tilapia (20, 21). Recombinant stFSH and stLH were able to elicit the release of E2 and 11-KT from ovarian and testis fragments, respectively (22). Recombinant cLH enhanced both E2

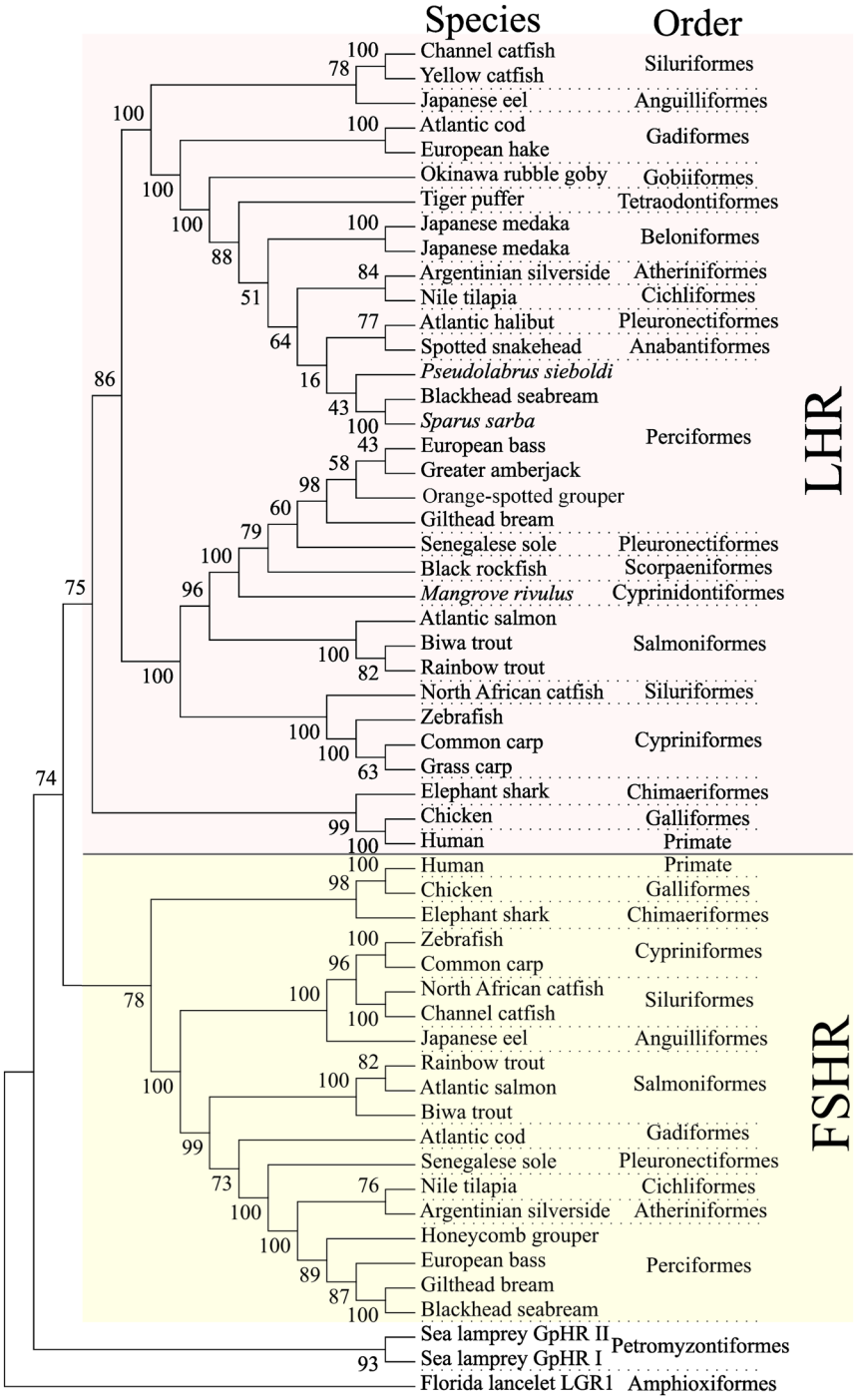

Figure 1
Maximum likelihood phylogenetic analysis of the LHCGR and FSHR from different organisms, the numbers in each branch represents bootstrap percentage values from 1000 replicates. Each order is highlighted in different color. Accession numbers for organisms represented here are detailed in Supplementary Table 1.

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and 17α, 20β, dihydroxy-4-pregnen, 3-one (DHP) secretion, and increased spawning success and fertilization rate when injected to mature female carps *in vivo* (23).

Isolation of carp ovarian follicles and steroid ELISA

Ovaries were collected immediately upon killing the fish and incubated as described previously (19). After rinsing, the medium was supplemented with either carp pituitary extract (CPE) or graded concentrations of homologous, recombinant cLHβα and cFSHβα (9, 19). The follicles were cleared in SERA solution (ethanol:formalin 40%:acetic acid; 6:3:1) (10), in order to determine the position of the germinal vesicle (GV) within the translucent oocyte. The number of oocytes at each stage was recorded: stage I: central GV; stage II: eccentric GV; stage III: migrating GV; stage IV: GV breakdown (GVBD) and stage V: ovulated eggs. Evaluation was done under a microscope (Olympus, CX31) with an image-analyzing device. E2 and DHP concentrations were analyzed by specific ELISAs (10, 19). Each experiment was performed on follicles harvested from the same female, and was repeated at least three times. All samples were analyzed in duplicate, and each ELISA plate contained a standard curve. The lower limit of detection was 1.56 pg/mL for either E2 or DHP. The intra- and inter-assay coefficients of variation were less than 7 and 11%, respectively.

Statistical analyses

Data are presented as means ± S.E.M. An unequal variance test was performed using JMP 7.0 software; in all data the variances of the samples were equal. The significance of differences between group means of expression levels was determined by ANOVA, followed by Tukey's test using GraphPad Prism 5.01 software (San Diego, CA, USA). To evaluate the correlation we used a Pearson coefficient in the correlation parameter in Prism. EC50 values of the receptors assays were calculated using log agonist versus response on a nonlinear regression curve using Prism.

Results

Molecular cloning, tissue distribution, and 3D models of the carp GTHRs

The cloned cFSHR cDNA (GenBank accession number MH726214) is 2010 bp in length, containing an ORF of 1950 bp that encodes a 650 amino acid protein, while the cloned LHCGR cDNA (GenBank accession number MH726213) is 2145 bp in length and encodes a protein of 696 amino acids (Supplementary Fig. 2). A putative N-terminal signal peptide of 20 and 19 amino acids were predicted for the FSHR and LHCGR, respectively by SignalP program analysis (http://www.cbs.dtu.dk/services/SignalP/). Like all other GpHRs, cFSHR and cLHCGR contain a very long N-terminal extracellular region (256 and 251 amino acids, respectively), a region consisting of seven transmembrane domains, and a C-terminal intracellular tail (Supplementary Fig. 2).

We examined a variety of tissues including the brain, pituitary, liver, intestine, spleen, fat, muscle, gills, retina, heart, kidney and gonads for the expression of cFSHR and cLHCGR. Not surprisingly, both FSHR and LHCGR were expressed at relatively high levels in the ovary and testis. Interestingly, both receptors were expressed in the pituitary, possibly connected to feedback mechanisms. LHCGR was highly expressed in the male pituitary compared to FSHR. The expression of LHCGR could be easily detected in the kidney, fat, spleen and gills, whereas FSHR was expressed in the retina, gills and front brain (Supplementary Fig. 3).

The evolutionary relationship of the cFSHR and cLHCGR to homologous GpHR sequences in other taxa was conducted by performing a phylogenetic analysis using the maximum likelihood method. The overall topology of the phylogenetic tree shows distinct LHCGR and FSHR clades, each with highly supported branches for sarcopterygian (mammalian, bird and amphibian molecules) and actinopterygian (fish) lineages. Both cFSHR and cLHCGR grouped with other species in the Cypriniformes like zebrafish, grass carp, and rohu, and with different species of catfishes like the channel and North American catfish (Fig. 1).

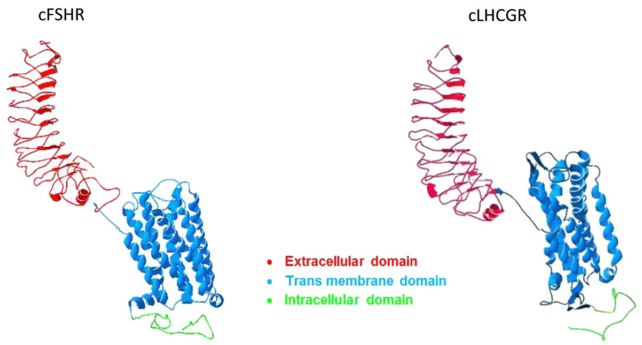

Figure 2
Three-dimensional models of the gonadotropin receptors that were calculated using the I-TASSER server and were visualized using SWIS-SPDV viewer. The extracellular domain is marked in red, the seven helices of the transmembrane domain are marked in blue, and the intracellular domain is colored green.

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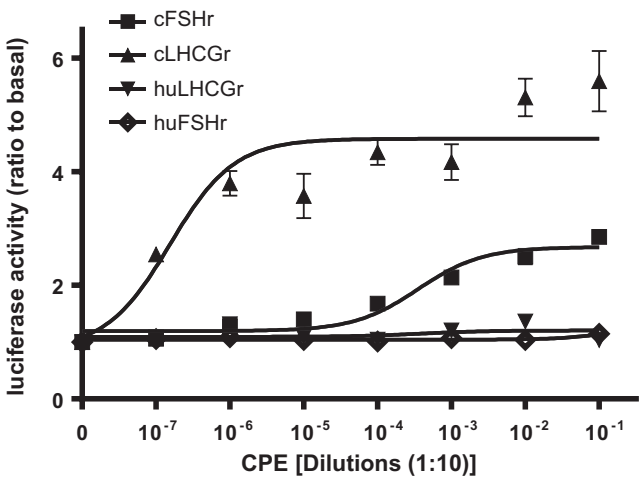

Figure 3
Carp and human gonadotropin receptors stimulated CRE-LUC activity in response to CPE. COS-7 cells were transiently co-transfected with reporter plasmid pCRE-LUC and carp gonadotropin receptors (cFSHR, cLHCGR) or human gonadotropin receptors (huFSHR, huLHCGR). Cells were stimulated for 6 h with decimal dilutions of CPE. Luciferase activity was determined and results are presented as a ratio to basal stimulation. Each assay was repeated at least three times. Data are presented as mean ± S.E.M. of a representative experiment, performed in triplicate.

Three-dimensional models of the carp GTHRs were conducted using I-TASSER. For each receptor the extracellular region and transmembrane region where modeled separately. The c-scores calculated by ITASSER for FSHR where 0.77 and −1.3 for the transmembrane and extracellular regions, respectively. For LHCGR the c-scores where −0.01 and −0.88 for the transmembrane and extracellular regions, respectively (Fig. 2).

Functional activation and characterization of carp FSHR and LHCGR

In order to test for the bioactivity of human and carp GTHRs by CPE we transiently expressed each receptor (cFSHR, cLHCGR, huFSHR or huLHCGR) in COS-7 cells, together with the reporter construct, pCRE-luc. CPE more effectively activated cLHCGR than cFSHR in terms of maximal response and effective dose. The human GTHRs were not activated by CPE (Fig. 3).

We next aimed to test the response of the cloned cFSHR and cLHCGR by stimulating transfected COS-7 cells with various doses of single-chain cGTHs (0.1–1000 ng/mL). Recombinant cLH activated cAMP signaling via cLHCGR in a dose-dependent manner (EC50 = 257.5 ng/mL). Moreover, recombinant cLH also activated cFSHR in a dose-dependent manner (EC50 = 279.2 ng/mL), while cFSH activated only its cognate FSH-R (EC50 = 176.2 ng/mL). Both gonadotropins activated cFSHR with a similar EC50 and maximal response, while activation of cFSHR was achieved mainly by cLH (Fig. 4 and Table 2).

To further characterize the novel cFSHR and cLHCGR, we tested the potential activation of intra- and inter-species gonadotropins. We used recombinant single-chain LH or FSH from a non-teleost Russian sturgeon (Acipenseriformes) (22), carp (Cypriniformes) (9), a recently evolved teleost (*Nile tilapia*), Perciformes; *Oreochromis niloticus* (20, 21), or human FSH or chorionic gonadotropin (hCG). To better compare between the activity of each ligand and the different receptors we ignored the EC50 of ligands that did not activate the receptor in a maximal fold change higher than 1.25 (marked as N/A in Table 2). cLHCGR-transfected cells responded to hCG, tLH and also tFSH, while cFSHR-transfected cells responded only to hFSH and tFSH (Fig. 5 and Table 2).

Expression of ovarian FSHR and LHCGR during sexual maturation in female carp

We next aimed to study the expression levels of both cFSHR and cLHCGR throughout the reproductive cycle of the female carp in parallel to ovarian development. At the primary growth stage (PG, follicle diameter 310–400 µm)

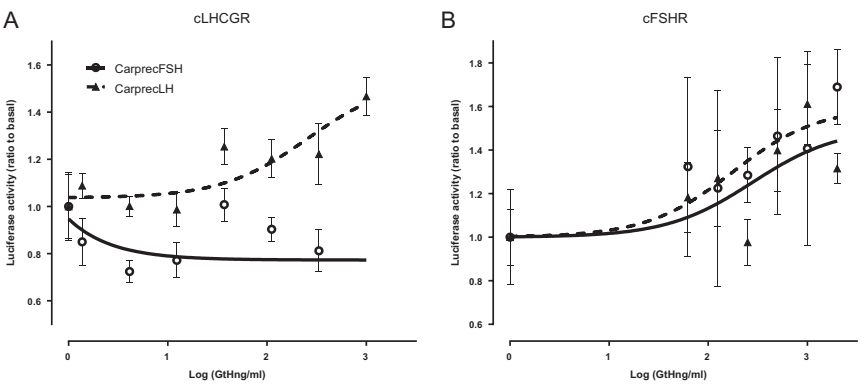

Figure 4
Carp gonadotropin receptors stimulated by carp (c) recombinant gonadotropins. COS-7 cells were transiently co-transfected with cLHCGR (A) or cFSHR (B) with the reporter plasmid pCRE-LUC. Cells were stimulated for 6 h with graded concentrations of recombinant cLH or cFSH. Luciferase activity was determined and results are presented as a ratio to basal stimulation. Each assay was repeated at least three times. Data are presented as mean ± S.E.M. of a representative experiment, performed in triplicate.

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Table 2 EC50 values (ng/mL) fit to a non-linear regression and the maximal response (fold change; MAX) of GTH-stimulated carp gonadotropin receptors (cFSHR, LHCGR) in receptor activation assays (Figs 4 and 5).

	cFSH		cLH		tFSH		tLH		hCG		hFSH		stFSH		stLH	
	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)	EC50 (ng/mL)	MAX (fold change)
cFSHR	176.2	1.7	279.2	1.6	351	1.5	N/A	1.2	N/A	1	843.7	1.3	N/A	1.2	4.5	1.3
cLHCGR	N/A	1.0	257.5	1.5	55.6	1.5	44.9	1.5	4.3	1.3	152.6	1.7	N/A	1.1	N/A	1.2

Stimulants with maximum fold change lower than 1.25 or without a sigmoid response were considered inactive and represented with N/A value.

both *fshr* and *lhcgr* had extremely low levels of expression. The expression of *fshr* significantly increased during early vitellogenesis, until follicle maturation. In contrast, the expression of *lhcgr* increased more gradually, becoming significant only at the mid-vitellogenic stage with the highest expression in mature follicles (Fig. 6).

Relationships between pituitary mRNA levels of pituitary GTHs, ovarian GTH-Rs, and GSI

Given the promiscuity of the gonadotropin receptors in the carp, it was interesting to test the correlation between mRNA levels of the gonadotropin ligands vs mRNA levels of the different GTH-Rs. *clhcgr* and *cfshr* mRNA levels significantly correlated with each other throughout the reproductive cycle. GSI was also highly correlated to both *clhcgr* and *cfshr* mRNA levels, while the mRNA of *lhb* in the pituitary was correlated to the mRNA levels of both GTHRs in the ovary. However, pituitary mRNA levels of *fshb* were significantly correlated only to ovarian *cfshr* (Table 3).

In vitro effect of carp recombinant GTHs on GVBD of preovulatory follicles

Taking in account the promiscuity of the cGTHRs, the next experiment was designed in order to answer the question whether cFSH and/or cLH can induce GVBD. We recently showed that the pituitary content of the various gonadotropins in the carp pituitary differ dramatically, when CPE contained milligrams cLH and only nanograms of cFSH (9). Thus, we used higher concentration of recombinant cLH (5–5000 ng/mL) than recombinant cFSH (1–1000 ng/mL) in order to induce GVBD *in vitro*. Interestingly, the maturational response significantly increased when increasing concentrations of both FSH and LH were used. Although no significant GVBD count difference was evident in response to graded dilutions of CPE, a trend of increase in the amount of mature follicles was observed (Fig. 7).

We further analyzed the steroid profile of the preovulatory follicles incubated with CPE or recombinant carp gonadotropins (Fig. 8). Varying dilutions of CPE did not affect DHP or estradiol levels. Increasing doses of cFSH significantly elevated DHP concentrations, and all the tested doses of cFSH decreased estradiol concentrations. High doses of recombinant cLH significantly increased DHP, but estradiol only demonstrated a negative trend with increasing recombinant cLH.

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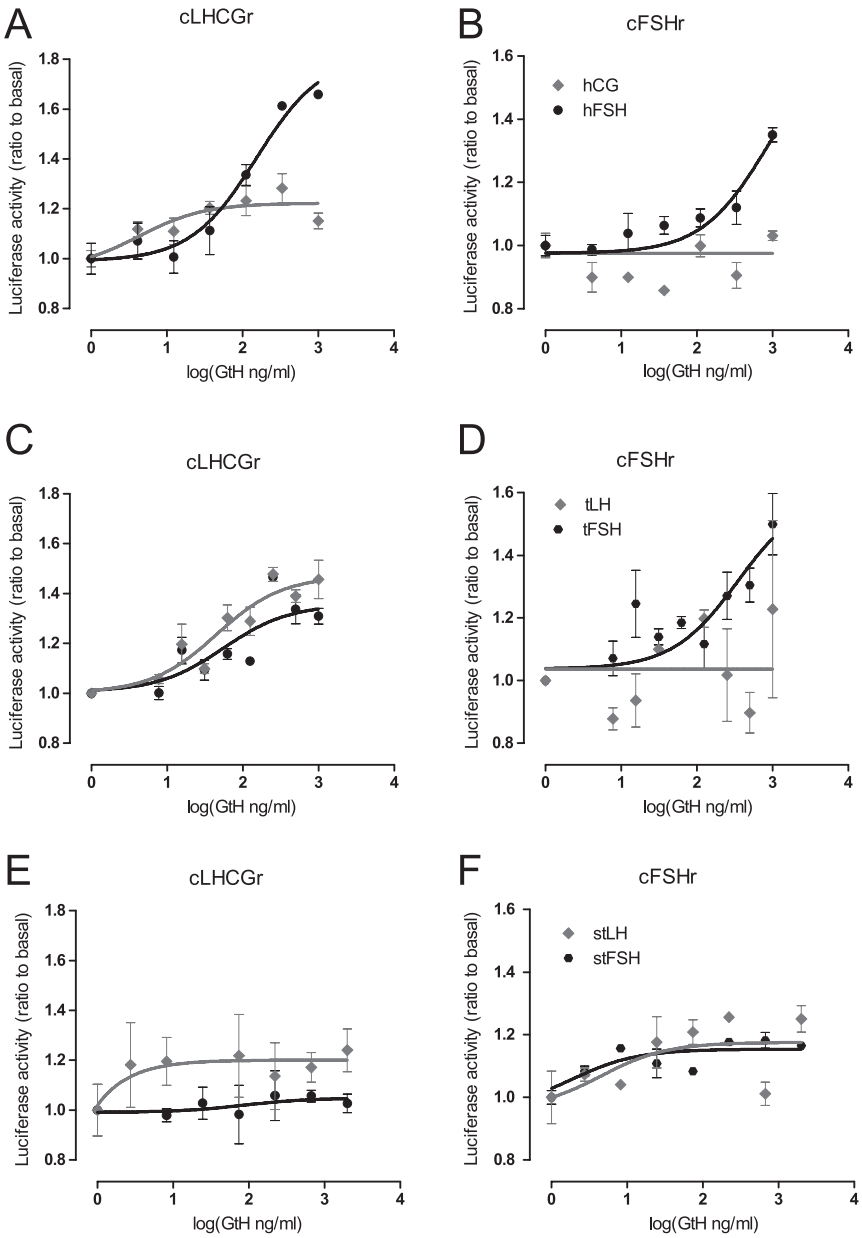

Figure 5
Carp gonadotropin receptors stimulated by recombinant gonadotropins of human (A and B), tilapia (C and D) or sturgeon (E and F). COS-7 cells were transiently co-transfected with cLHCGR (A, C and E) or cFSHR (B, D and F) and with the reporter plasmid pCRE-luc. Cells were stimulated for 6 h with graded concentrations of tilapia (t), human (h), and sturgeon (st) recombinant gonadotropins. Luciferase activity was determined and results are presented as a ratio to basal stimulation. Each assay was repeated at least three times. Data are presented as mean ± S.E.M. of a representative experiment, performed in triplicate.

Discussion

The duality of fish gonadotropins and their receptors was established 25 years ago (24), but the evolutionary trend of specific FSHR and LHCGR activation by the gonadotropins, cognate or heterologous, in different fish species is unknown. In the current study, we cloned the full-length cDNAs for FSHR and LHCGR of the common carp and analyzed their expression profiles during the female reproductive cycle. We additionally studied the ability for homologous and heterologous GTHs to activate cAMP signaling via the cLHCGR or cFSHR in an attempt to characterize a functional evolutionary model that summarizes the permissive actions of fish GTHRs.

The current accepted model of the specific, distinct functions of FSH and LH in fish emerged from studies on salmonids, which spawn once per life or in a year and have synchronously developing ovaries. In the salmon, FSH and LH are discretely and temporally secreted during the reproductive cycle, where FSH is mainly secreted during the long phase of vitellogenesis and LH is secreted during final oocyte maturation and ovulation (25). Additionally, female Amago salmon FSHR mRNA expression was high during the early stages of vitellogenesis and gradually decreased as gonadal maturational ensued, while LHCGR expression peaked during final maturation (26). Similar seasonal patterns were reported in the oogenesis of another synchronous

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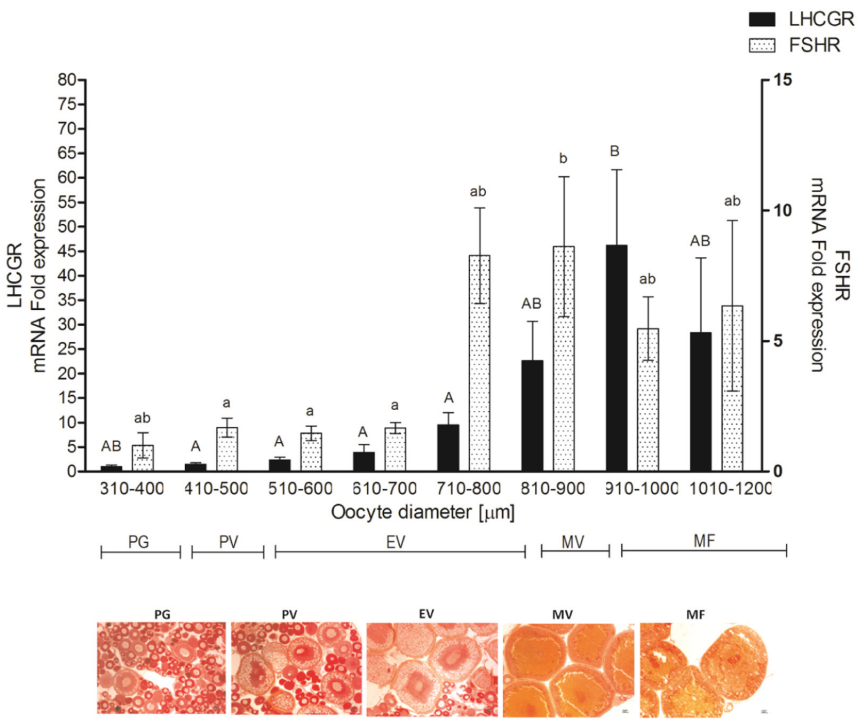

Figure 6
Changes in FSHR and LHCGR mRNA expression in the ovary at various stages of gonadal maturation as determined by real-time PCR. Fold expression was normalized to the geometric mean of EF-1α and β-actin by the comparative threshold cycle method (ΔCT). Results are shown as mean ± S.E.M. (n = 3–14). Means marked with different capital letters have statistically different LHCGR expression, and means marked with different lower-case letters differ significantly in FSHR expression ($P < 0.05$). The ovarian developmental stage of each fish was determined with hematoxylin and eosin-stained histological sections of carp ovary in different maturation phases according to oocyte diameter; the stages are represented in the bottom of the graph. The primary growth (PG) stage contains mainly prenuclear follicles with big central nucleus. The previtellogenic (PV) stage contains some larger follicles with cortical alveoli in their cytoplasm. The early vitellogenic (EV) stage contains large follicles with oil vacuoles and yolk granules accumulated in the cytoplasm. The mid-vitellogenic (MV) stage contains follicles where the germinal vesicle starts its migration to the periphery and the yolk granules become yolk plates. Mature follicles (MF) went through the dehydration stage where the follicle reaches to its maximum growth.

spawner, the channel catfish (27). However, the carp is a total spawner; i.e., when maturation of the gonads begins, all the eggs or sperm develop synchronously. Hormonal levels, histological observations and gene expression revealed that for the carp, oocytes during the cortical alveolus stage appeared when follicle diameter was 410–500 µm, vitellogenesis then started and was completed when follicle diameter was 810–900 during February. The spawning season was between April and June, which corresponded with a significant increase in the GSI, DHP, and estradiol levels (9, 14). However, although the carp is a total spawner, mRNA levels of both FSHR and LHCGR were low during vitellogenesis and increased concomitantly toward oocyte maturation, where FSHR level increased earlier than LHCGR. In the ovary of the Rohu (*Labeo rohita*), an Indian carp which is also a total-spawner, *fshr* gene expression was also enhanced from the primary growth stage to the pre-spawning phase and maintained at the same level during the spawning phase, while *lhcgr* expression enhanced from the primary growth stage to pre-spawning phase, reaching a peak at the spawning phase (28). This is in accordance with our previous results showing that LH pituitary content and mRNA levels were low at pre- and early vitellogenesis, reached a peak in mid-vitellogenic ovary, and a peak of LH content in fully grown ovarian follicles (9). However, no significant change occurred in FSH pituitary content and mRNA levels between vitellogenic fish and fish at the

Table 3 Correlation tests between the mRNA levels of the gonadotropin receptors and the mRNA levels of the gonadotropins (9) and GSI.

	LHβ			FSHβ		cLHCGR	GSI	
	vs FSHβ	vs cLHR	vs cFSHR	vs cLHR	vs cFSHR	vs cFSHR	cLHR	cFSHR
Number of XY pairs	39	36	39	38	39	38	72	76
Pearson *r*	0.360	0.410	0.232	0.046	0.310	0.547	0.638	0.479
P value (two-tailed)	0.0244	0.013	0.1543	0.7844	0.0119	0.0004	< 0.000	< 0.000
P value summary	a	a	ns	ns	a	b	b	b
Is the correlation significant? (alpha = 0.05)	Yes	Yes	No	No	Yes	Yes	Yes	Yes
R squared	0.130	0.168	0.054	0.002	0.159	0.300	0.406	0.406

Correlation was performed using GraphPad Prism program v5. [a]$P<0.05$; [b]$P<0.01$.

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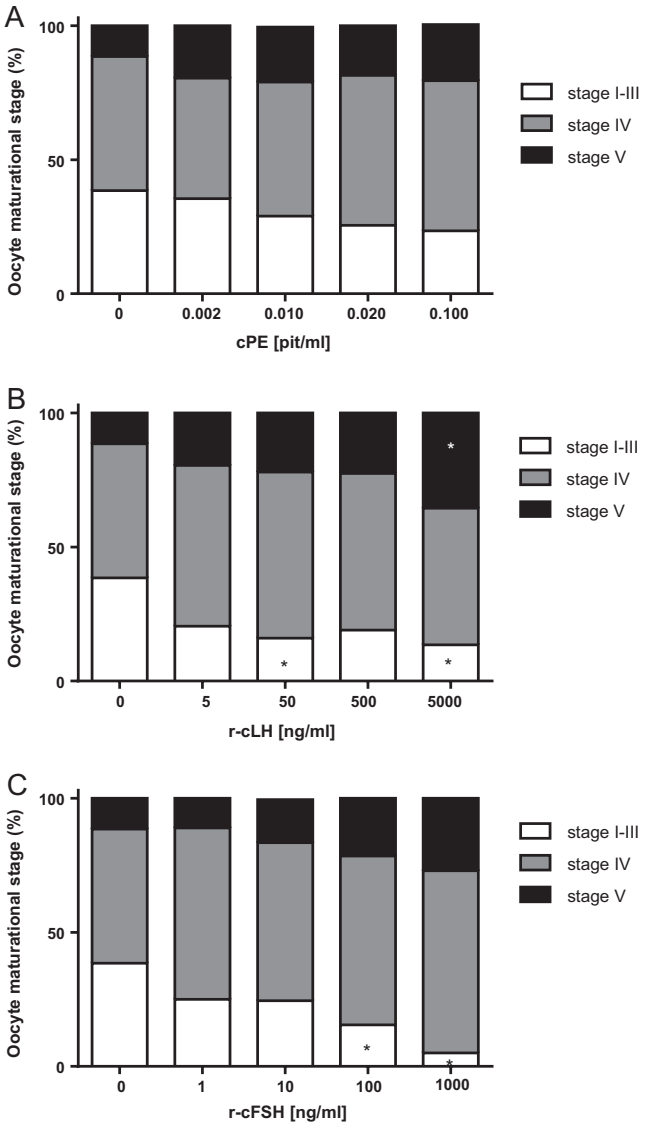

Figure 7
Induced maturation progress of common carp ovarian follicles after 12-h stimulation in the germinal vesicle break down assay applying carp pituitary extract (CPE with a concentration of 0.49 mg/pit cLH and 4.79 ng/pit cFSH) (A), recombinant cLH (r-cLH) (B) and recombinant cFSH (r-cFSH) (C). Data are presented as mean ± S.E.M. and statistically significant differences between treatments and control are highlighted with * ($P < 0.05$).

final stages of maturation (29). In goldfish too, LH was found to be present throughout the reproductive cycle, leading to the conclusion that mainly LH was involved in regulating gonadal steroidogenesis, gametogenesis, and ovulation (30). In line with these data, increases in *fshr* gene expression have been correlated with later events of the reproductive cycle including the oocyte maturation and ovulation in the rainbow trout (31), zebrafish (32) and tilapia (29). These data suggest that piscine gonad development and maturation depends on a specific gonadotropin profile that does not reflect the temporally distinct dual-gonadotropin model observed in salmonids or mammals.

DHP is known as the maturation-inducing steroid in most fish species (reviewed in (1)). One of the major endocrine events associated with termination of vitellogenesis and meiosis resumption is an LH-driven shift in the ovarian follicle steroidogenic pathway from the production of predominantly estradiol during vitellogenesis to the production of DHP (33). This switch is associated with decreased expression of cytochrome P450 aromatase (34). We show here that both recombinant cFSH and cLH successfully induced GVBD in the carp. Similar results were found in the walking catfish where both semi-purified LH and FSH induced GVBD *in vitro* (35). Moreover, DHP levels significantly increased in response to both gonadotropins, while estradiol decreased only in response to recombinant cFSH. We previously showed that injection of the resolving high dose of CPE resulted in a shift in the main ovarian steroid from estradiol to DHP (10). Incubation of carp ovarian fragments with both FSH and LH from various piscine sources also resulted in an increase of both estradiol and DHP secretion (19). The acquisition of follicular maturational competence that naturally occurs during post-vitellogenesis in the carp also corresponds to an increase of follicular responsiveness to both gonadotropins. Taken together, these data suggest that FSH induces DHP production/secretion, not through binding to the LHCGR, but rather by cognate binding to FSHR, which results with DHP secretion.

It is accepted that receptor binding domains and their ligands experience co-evolution, where reciprocal restrictions are applied over evolutionary time (36). Previously, fish GTH specificity has been viewed from too large of a vista under the assumption that evolutionary rate and polyploidization of the genome contribute to permissive GTHR activation. The gonadotropin beta subunits are encoded by paralogous genes descending from a common ancestor and share about 40% sequence identity (for both human and carp). The corresponding receptors are also encoded by paralogous genes and, accordingly, they also display about 40% sequence identity in their hormone-binding ectodomain. In contrast, the effector domains of the receptors share higher sequence identity (about 70% for both human and carp), which suggests that they fulfill essentially the same function – activation of $G\alpha_s$. The hypothesis that earlier evolved lineages have a more fundamental GTHR sequence that would facilitate the activation of any later evolved GTH has received mixed support (19, 37, 38).

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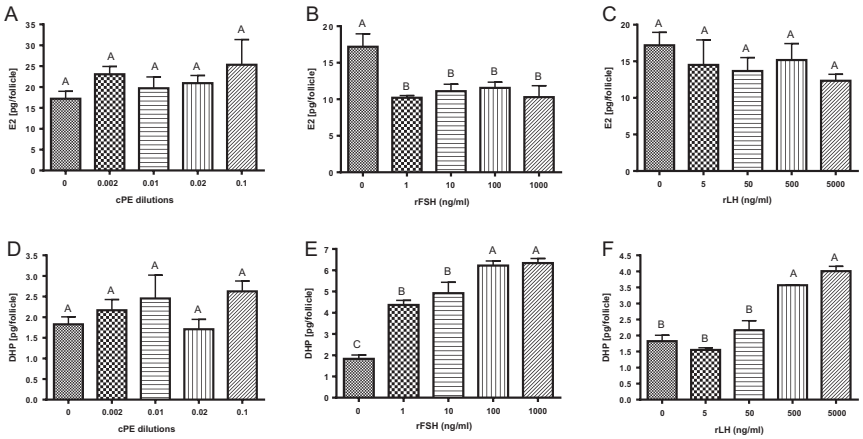

Figure 8
Effects of different doses of carp pituitary extract (CPE) with a concentration of 0.49 mg/pit of cLH and 4.79 ng/pit of cFSH (A and D), recombinant cFSH (r-cFSH; B and E) and recombinant cLH (r-cLH; C and F) on E2 (A, B and C) and DHP (D, E and F) secretion *in vitro* by intact preovulated ovarian follicles. Each point represents the mean ± S.E.M. of follicles from three different fish per treatment group, with three replicates ($n = 9$). Means marked by different letters differ significantly ($P < 0.05$).

However, fish receptors respond to mammalian gonadotropins, but human receptors are not activated by fish ligands (39) (Fig. 3). Sea bass FSHR was specifically stimulated by bovine FSH, while sea bass LHCGR could be activated by both bovine LH and FSH, demonstrating not only inter-species activation, but also cross-reactivity of the GTHs (40). This is also true of the eel LHCGR, which could be activated by hFSH, hCG, and even trout FSH (39). In addition to inter-species cross activation, in zebrafish and African catfish, homologous LH binds both GTHRs, whereas FSH binds only its own cognate receptor (41, 42, 43) (Fig. 9). Our results showed a certain degree of inter-species cross-reactivity, where cLHCGR was activated by tFSH, tLH, hCG and hFSH, and cFSHR was activated by tFSH, hFSH, and stLH. Both cGTHRs, along with other teleost species, permit inter-species GTH cross-reactivity,

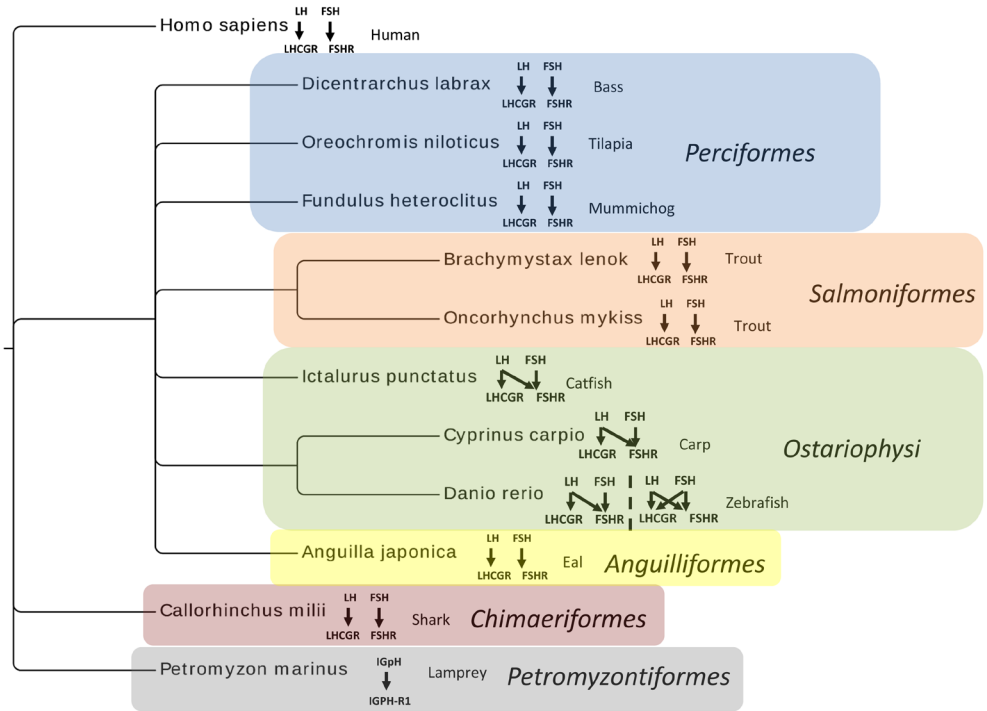

Figure 9
A model describing the specificity of gonadotropins binding to their cognate receptors in representative species of fish from different piscine orders. The taxonomic tree and the selection of different order groups was done using *Taxonomy browser* (NCBI) and the design was performed in *Evolview* (http://www.evolgenius.info/evolview). This model was created using studies where homologous recombinant gonadotropins were used: (50) for the rainbow trout (*Oncorhynchus mykiss*); (32) for zebrafish, (42, 51) for African catfish (*Clarias gariepinus*), (40, 52) for Sea Bass (*Dicentrarchus labrax*); (53) for mummichog (*Fundulus heteroclitus)*; (39) for eel, tilapia and human; (54) for Atlantic halibut (*Hippoglossus hippoglossus*); (55) for amago salmon (*Oncorhynchus rhodurus*); (56) for Manchurian trout (*Brachymystax lenok*); and (38) for elephant shark (*Callorhinchus milii*).

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implicating a functionally basic property of these GTHRs. However, these functional observations are complicated by a complex evolutionary history of the teleosts which may or may not have a demonstrated trend. In the view of the expansive teleost radiation, it seems most appropriate to conclude that permissive GTHR activation is a consequence of lineage-specific, biologically relevant events, and not a less-evolved theme for the entire infraclass (Fig. 9).

The response of the cLHCGR to the homologous pituitary extract was twice that for cFSHR. This can be due to the higher levels of cLH in the pituitary (9), and also to the higher number of LH-secreting cells in the pituitary of mature fish as was shown for zebrafish and tilapia (44, 45).

The cLHCGR and cFSHR ECDs have a more neutral concave surface potential, but differ in the ECD terminals where cLHCGR has strong basic and acidic propensities on the N- and C-terminal sides of the ECD, respectively, and the cFSHR has a weaker acidic N-terminal propensity that covers more surface than cLHCGR and a weakly basic C-terminus. We showed that recombinant cLH and cFSH both activate cFSHR, but cLHCGR was only activated by its cognate ligand. We also observed heterologous activation of cLHCGR with tilapia and human FSH and cFSHR with sturgeon LH. Interestingly, stFSH and hCG did not activate cLHCGR and cFSHR, respectively, perhaps alluding to some species-specific binding and activation determinants. The generally weaker proximal charges of the cFSHR ECD could permit more permissive initial binding, but recognition specificity is not equivalent to functional specificity. The LRR domain is involved in initial recognition and binding to the receptor; however, the signal specificity domain (SSD; 'hinge') and the extracellular loops of the TMD bridge the gap between hormone binding and receptor activation (46). The SSD is located between the last repeat of the LRR domain and the first helix of the TMD, and provides a high-affinity binding site in addition to the LRR domain (47). It is characterized by having two highly conserved cysteine-rich boxes (Cb-2 and Cb-3) that form important disulfide bridges and key determinant residues for hormone binding and receptor conformational changes (48). Teleost FSHRs lack approximately 30 amino acids between conserved cysteine 3 and 4 of Cb-2/3 compared with human and chicken FSHR, resulting in a shorter hinge loop that could influence the degree of secondary ligand binding and activation. This same region in the LHCGR of teleosts is variable in size, but is longer than their FSHR counterparts. A conserved sulfated tyrosine residue (Y335 of hFSHR, Y331 of hLHCGR) found in the SSD is indispensable for binding and subsequent activation (49). The principal binding of a GpH to the LRR domain causes a low-structured loop of the SSD containing the sulfated tyrosine to move into an exposed hydrophobic pocket between the alpha and beta subunits of the GpH (47). cLHCGR and FSHR do have conserved tyrosine residues in this region (Y346 and Y311, respectively), but there are no data addressing the presence of a sulfate moiety on these residues in fish. The differences in overall electrostatic potential of the ECD, length of the SSD, and various binding residues between cLHCGR and cFSHR contribute to the permissive actions of these receptors, but further experimentation will be required in order to determine the exact factors that govern GTH binding specificity in fish.

We have demonstrated here that the specific role of the two gonadotropins might be different in carp than in the accepted model. Our data support the hypothesis that fish gonadotropin and receptor expression and interaction profiles are different than those of other vertebrate lineages due to the vast radiation of teleosts and their reproductive strategies in diverse ecological niches. Furthermore, we suggest that the permissive activation of the fish gonadotropin receptors, which was previously attributed to all fish species, is mainly a characteristic of *Ostariophysi* species.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0389.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie Grant Agreement no 642893 – IMPRESS. KNH was supported by BARD, the United States – Israel Binational Agricultural Research and Development Fund, Vaadia-BARD Postdoctoral Fellowship Award No. FU-561-2017.

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Received in final form 29 September 2019
Accepted 2 October 2019
Accepted Preprint published online 3 October 2019