



Regulation of steroid production and key genes in catfish *Heteropneustes fossilis* using recombinant gonadotropins

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Abstract The two gonadotropins, FSH and LH, stimulate growth and development of the gonads through gonadal biosynthesis of steroid hormones and growth factors. To date, cDNA sequences encoding gonadotropin subunits have been isolated and characterized from a large number of fish species. Recently, we successfully cloned and characterized gonadotropins (LH β , FSH β , and GP α) from the pituitary glands

of the catfish, *Heteropneustes fossilis*. In the present study, we describe herein the production of recombinant stinging catfish, *H. fossilis* (*hf*) FSH (rhfFSH) and LH (rhfLH) using the methylotrophic yeast *P. pastoris* expression system. We further explored the hypothesis that the recombinant gonadotropins can modulate the hypothalamus-pituitary-ovarian (HPO) axis genes (*avt*, *it*, *gnrh2*, *kiss2*, and *cyp19a1a*) and regulate their transcriptional profile and steroid levels in relation to their annual developmental stage during preparatory and pre-spawning phases under in-vitro conditions. We found that the different concentrations of recombinant rhfFSH and rhfLH significantly stimulated E2 levels in the preparatory and prespawning season, and also upregulated gonadal aromatase gene expression in a dose dependent manner. Our results demonstrate that the yeast expression system produced biologically active recombinant catfish gonadotropins, enabling the study of their function in the catfish.

Highlights

- Catfish recombinant LH β and FSH β production using a *Pichia pastoris* yeast expression system
- Follicular maturation shown to be season dependent with respect to rhfLH/rhfFSH
- 17 β -Estradiol and testosterone production found to be stimulated by rhfLH/rhfFSH in a concentration dependent manner
- rhfLH/rhfFSH promotes differential expression of genes *gnrh2*, *kiss2*, *avt*, *it*, and *cyp19a1a*

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Introduction

Aquaculture is constantly seeking ways to enhance reproductive control. Reliable protocols are established to manage the reproduction of adult fish and

secure a dependable supply of juveniles. Given that the reproductive ability of many commercial species is hindered in captivity, exogenous hormonal therapies have been developed to promote aquaculture production. (Aizen et al. 2017; Ramos-Júdez et al. 2022).

The pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) play a central role in the gonadal development of vertebrates (Pierce and Parsons 1981). Through binding to membrane receptors, FSH stimulates follicular development in the ovary and gametogenesis in the testes, while LH stimulates processes leading to the final oocyte maturation and ovulation in females, and spermiation in males (Levavi-Sivan et al. 2010; Sambroni et al., 2013). Over the last 3 decades, the advances in molecular sequencing and cloning have enabled the isolation of numerous fish gonadotropin sequences, representing more than 20 teleost orders. This has enabled the production of recombinant hormones (mainly FSH and LH) using a variety of expression systems starting with insect cells, vertebrate cells, and now primarily yeast cells (Peter Dennis et al. 2020) replacing the need for isolating native hormones (Molés et al., 2020).

The use of recombinant gonadotropins in fish can help to better understand fish reproductive endocrinology, especially in fish species that are new to aquaculture, or to elucidate the role of these hormones. Numerous studies have shown that the recombinant FSH and LH induce steroid secretion *in vivo* and *in vitro*, both in males and females (Molés et al. 2020).

The catfish *H. fossilis* (stinging catfish) is a freshwater airbreathing species distributed in Southeast Asian countries in ponds, lakes, streams, and other natural bodies of water. It is a seasonal breeder with a distinct annual reproductive cycle. Gonadal recrudescence occurs coincident with the rise in photoperiod and temperature (March to June); spawning is induced by the monsoon rainfall (July–August), which is preceded by prolonged gonadal quiescence from September to February. Seasonal changes in reproductive parameters including gonadosomatic index (GSI), gonad histology and steroid hormones, LH levels, and GtH subunit mRNA levels have been previously described (Lamba et al. 1983; Tharakan and Joy 1996; Acharjee et al. 2015). It has been well established that stinging catfish gonadotropins (FSH and LH) stimulate steroidogenesis and that the steroid hormones are the tertiary regulators controlling specific events

in gametogenesis and spawning (Vischer et al. 2003; Zmora et al. 2003, 2007; Chourasia et al. 2022). The ovarian cycle is conventionally divided into previtellogenic, vitellogenic (primary and secondary growth), and post vitellogenic maturational stages (Khan and Thomas 1999). 17 β -Estradiol (E₂) is the major estrogen synthesized in the vitellogenic phase (Fostier et al. 1983; Babin et al. 2007). Upon the completion of vitellogenesis, E₂ synthesis is decreased and under appropriate spawning conditions for the induction of a preovulatory LH surge the post vitellogenic follicles initiate maturational activity (resumption of meiosis) under the influence of a maturation-inducing hormone (MIH) (Chourasia et al. 2022).

Hormonal therapies have been developed in the fish in order to overcome the reproductive bottleneck at the level of the pituitary gland, focusing almost exclusively on spawning induction (Mylonas et al. 2010). In general, catfish do not breed in captivity in India, and the current practices of achieving reproductive success in captivity are significant to reduce pressure on wild fish stocks, provide seed for restocking bodies of water with declining populations, and restore and preserve endangered endemic species. It has been reported that by gonadotropin intervention, the sexual cycle can be advanced, and brooders can be raised beforehand for artificial breeding.

In the catfish *H. fossilis*, *gnrh2*, transcripts are expressed extensively in the brain, suggesting varied region-specific functions. Brain *gnrh2* expression is modulated by feedback actions of gonadal steroids (Chaube et al. 2019). *gnrh2* transcripts are also expressed in the gonad, indicating a paracrine/autocrine role in gonadal function. The *cyp1a* and *cyp1b1* genes are highly expressed in the liver, brain, and ovary, with the ovary and brain *cyp1a* and *cyp1b1* showing seasonal variations with the highest expression in the spawning phase.

The ovarian *cyp1a* and *cyp1b1* transcripts were localized in the endocrine follicular layer. Gonadotropin and 2-OHE₂ stimulated gene expression during the periovulatory phase, supporting the involvement of Cyp1 proteins in human chorionic gonadotropin (hCG)-induced E₂ hydroxylation and formation of catechol estrogens, events which are critical in the regulation of FOM in *H. fossilis*.

It has been reported that in catfish *H. fossilis avt*, the basic nonapeptide hormone is secreted

by the nucleus preopticus in the hypothalamus and released from the pituitary into circulation as a neurohormone. Singh and Joy (2010) demonstrated that the oocytes of the freshwater catfish *H. fossilis* undergo hydration (maximum increase in water content) during meiotic maturation in-vitro induced with hCG or vasotocin (VT) (Singh and Joy 2010). They also showed that the hCG and ovarian steroids increase the production of VT in ovarian follicles in-vitro and VT has elicited steroidogenic activity thus indicating direct or indirect involvement in ovarian functions (Singh and Joy 2009b, 2009a; Chaube et al. 2011). Furthermore, it was shown that like hCG, VT and isotocin (IT) have differential effects on ovarian steroidogenesis and may be involved directly or indirectly in ovarian functions, as a paracrine/autocrine factor or a neurohormone (Singh and Joy 2009b). Seasonal variations and alterations due to photo–thermal changes and steroids in the expression of these genes are also suggestive of a role in reproduction (Chaube et al. 2019, 2020a, 2021). In catfish, *kiss2* is an important regulator of the brain-pituitary-gonadal-endocrine axis, and in habenular and optic tectum functions (Chaube et al. 2020b). Recent studies in *H. fossilis* have shown that the kiss peptides can act at the level of brain, pituitary and ovary to modulate reproduction, highlighting the aquaculture potential of kisspeptins in fish and make the peptides promising agents for induced breeding (Chaube et al. 2022).

The catfish is cultured in Southeast Asian countries as an important food source and GtH and GnRH-based artificial breeding techniques have been successfully employed in this species (Alok et al. 1993; Acharjee et al. 2017).

In the present study, we herein describe the production of recombinant stinging catfish, *Heteropneustes fossilis* (*hf*) FSH (rhFSH) and LH (rhLH) using the methylotrophic yeast *P. pastoris* expression system. We further explored the hypothesis that the recombinant gonadotropins can modulate the HPO axis genes (*avt*, *it*, *gnrh2*, *kiss2*, and *cyp19a1a*) and regulate their transcriptional profile and steroid levels in relation to their annual developmental stage, during preparatory and pre-spawning phases under in-vitro conditions.

Material and methods

Fish

Stinging catfish, *Heteropneustes fossilis* (*hf*), were collected from fish markets in Varanasi during the preparatory (April, 2.2 ± 0.8 g%, 20 ± 2 °C) and pre spawning (June, 3.2 ± 1.2 g%, 23 ± 2 °C) phases of the annual reproductive cycle and were reared under standard aquaculture conditions. They were maintained in the laboratory for 48 h under natural photoperiod and temperature to overcome the stress of transport, and fed goat liver ad libitum. All experimental procedures comply with the animal care and use guidelines of the Banaras Hindu University for experimentation in animals and all care (permit no: 1802/GO/RE/S/15/CPCSEA) was taken to prevent cruelty of any kind.

Chemicals and reagents

For recombinant study

All chemicals and reagents for the work with the *P. pastoris* system were as described in the multi-copy Pichia Expression Kit (Invitrogen) protocols.

For gene expression study

Gene-specific primers of *H. fossilis* *gnrh2*, *avt*, *it*, *kiss2*, *cyp19a1a*, and β -actin were designed and custom-synthesized by Integrated DNA Technologies (IDT), Hyderabad, India. RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany), Revert-Aid H minus first strand cDNA synthesis kit (Fermentas, Foster, CA, USA), DNase I, RNase-free (Ambion, Foster, CA, USA), RNAlater (Ambion, Foster, CA, USA), 2 x PCR Mastermix (Fermentas, Foster, CA, USA), VeriQuest™ SYBR Green qPCR Master mix with ROX (Affymetrix, Inc. Cleveland, Ohio, USA) were purchased through local suppliers. Tricaine methane-sulfonate (MS 222), estradiol-17 β (E2), TRIS base, HEPES and EDTA were purchased from Sigma-Aldrich, New Delhi, India. Agarose. Leibovitz L-15 medium, and other chemicals were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Estradiol-17 β and testosterone ELISA kits (Labor Diagnostika Nord GmbH & Co. KG, Germany) were purchased through local suppliers.

Recombinant gonadotropin production

Synthetic FSH and LH genes were ordered from GeneScript (Piscataway, USA). The genes contained a linker containing a His-tag (6xH) and the codon usage was optimized to the codon bias of *P. pastoris* to facilitate higher expression rates. All recombinant proteins used in this work were cloned into the *EcoRI*-*NotI* sites of the pPIC9K expression vector. Briefly, the synthetic construct was based on the cDNA encoding the mature secreted form of hfFSH β (GenBank Accession No. KF573627) or hfLH β (GenBank Accession No. KF573628) and hfFSH/LH α (GenBank Accession No. KF573626) (Acharjee et al. 2015) without their cognate signal peptide. The mature protein-coding sequences (beta (FSH or LH) and alpha subunit; GenBank Accession No. beta FSH: AHB20169.1, LH beta: AHB20170.1, FSH/LH alpha: AHB20168.1) were joined to form a fusion gene that encodes a “tethered” polypeptide in which the beta subunit forms the N-terminal and the alpha chain forms the C-terminal. A “linker” sequence of amino acids (GSGSHHHHHHSGSGS) was placed between the beta subunit and the alpha subunit to assist in the chimerization of the subunits, with the six-His tail placed in the middle of the linker to enable purification of the recombinant protein (Fig. 1A).

The recombinant single chains were sub-cloned into pPIC9K expression vector. Prior to yeast transformation, the pPIC9K vector containing the synthetic gene was linearized with *Sal I* to obtain Mut+ (methanol utilization plus: refers to the wild-type strains’ ability to metabolize methanol as sole carbon source) transformants. The *Sal I*-linearized expression cassette was then transformed into the host *P. pastoris* SuperMan5 strain (*his*⁻), aGS115 (*his4*-) variant with the alpha 1, 2-mannosidase from *Trichoderma reesei* regulated by the GAP promoter on a plasmid with the blasticidin resistance gene disrupting the *Och1* gene in the SuperMan5 genome, by electroporation. This resulted in insertion of the construct at the *AOX1* locus of *P. pastoris*, generating a His⁺ Mut⁺ phenotype. 200 Mut+ transformant colonies were screened by resistance test with 0.5 to 2 mg/ml antibiotic (G418 geneticin). Ten His/Mut+ clones were chosen and cultured for small-scale production in a shaker flask for 1 day (growth phase) in BMG at 28 °C. Cells were harvested by centrifugation, resuspended, and cultivated for 3 days (induction phase) in BMM medium. The proteins were expressed in a shaker flask and harvested after induction by methanol. Recombinant proteins were purified using nickel-nitrilotriacetic acid-agarose (Ni-NTA; Qiagen). Using the methylotrophic yeast *P. pastoris*, hfFSH, and hfLH were produced as

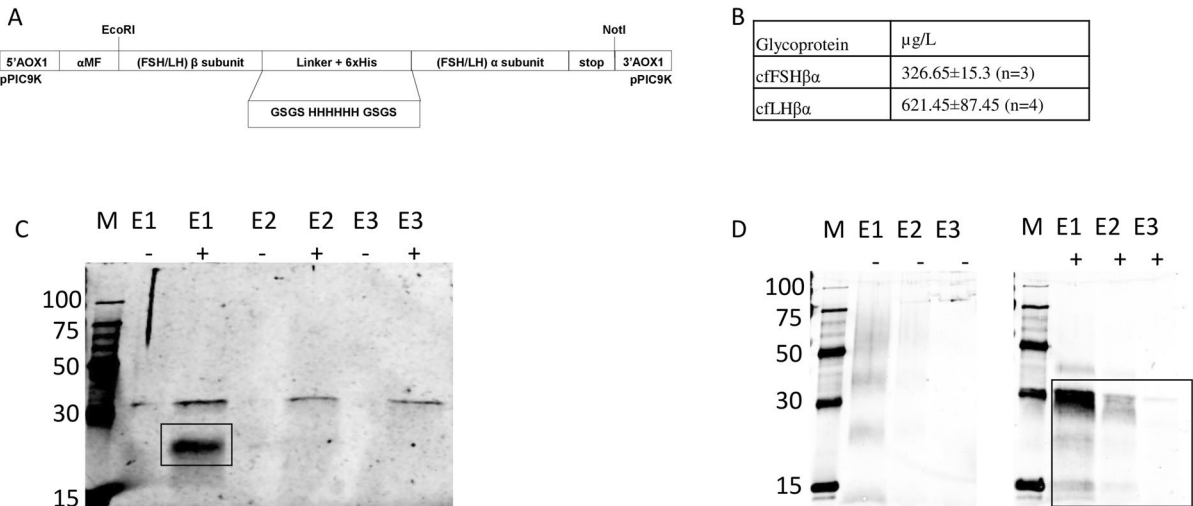


Fig. 1 **A** Schematic representation of the expression cassette in pPIC9K vector for each gonadotropin. **B** 1 L production summary for each gonadotropin, data is presented as Mean \pm SEM, $\mu\text{g/L}$. Characterization of *Pichia*-expressed recombinant catfish FSH $\beta\alpha$ **C** and LH $\beta\alpha$ **D** by western-blot analysis and

deglycosylation. Supernatants of transformed *Pichia* cultures were separated by 15% PAGE-SDS (E1–3—elution 1–3) and immunoreacted with antibodies raised against anti-His. For deglycosylation analysis, denatured and reduced proteins were incubated with (+) or without (–) N-glycosidase F

biologically active, single-chain polypeptides (Aizen et al., 2007a) by using the *Pichia* Expression kit (Life Technologies Corp.)

Western blot analysis

Reduced or nickel-purified samples, both non-deglycosylated and deglycosylated [by PNGase F (New England Biolabs) from culture supernatants] were electrophoresed on 15% Tris/glycine gels. Gels were blotted onto nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) and blocked with 3% BSA in TBS-T. Recombinant gonadotropins were visualized with an antibody against the His-tag (QIAexpress anti-His antibodies; Qiagen). When using the anti-His antibody, the membranes were treated according to the manufacturer's recommendations (1:2000 dilution). Membranes were next incubated with IRDye® 800CW Goat anti-Mouse secondary antibody for 1 h at room temperature. After washing, all membranes were analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

In vitro bioassay

Briefly, gonads from 24 mature *catfish females* (12 from each reproductive phase; mean \pm SEM, 60–80 \pm 9.8 g of body weight [BW]); gonadosomatic index [GSI] [i.e., gonadal weight percentage of BW], 2–10% \pm 2.5%), were divided into uniformly sized fragments (of about 90–100 mg each). The fragments

were washed 3 times for 5 min in a 24 well-culture plate at 28 °C in the presence of Leibovitz's L-15 Medium (Sigma). The ovarian fragments were then rinsed, and the medium was replaced with the same medium with or without the treatment to be tested. Stimulation with each gonadotropin, at graded doses (1000, 500, 250, 125, 62.5, 31.25 ng/ml, and hCG (100 ng/ml)), was continued for 15–20 min. These incubations were performed in triplicate wells per treatment ($n = 3$) and qPCR was done independently for each sample.

qRT-PCR assay

The qRT-PCR assay was conducted according to the procedure adopted by us previously (Chaube et al. 2017). The stored tissues (ovarian fragments) were processed for total RNA extraction using the RNeasy mini kit (Qiagen). RNA purity was checked by calculating A260/A280 ratio and samples with a ratio of 2.0 or higher were used. Genomic DNA contamination in the RNA preparations was checked by using non-reverse transcribed samples as templates and by treating the samples with DNase I before the first strand cDNA synthesis. Total RNA (5 μ g) was reverse transcribed using random hexamer primers and Revert Aid M-MuLV reverse transcriptase in a 20 μ L reaction volume (first strand cDNA synthesis kit, Fermentas), using the manufacturer's protocol. Quantitative PCR assays were performed in triplicate for different samples using specific forward and reverse primers of target genes (Table 1) and VeriQuest™ SYBR

Table 1 Primers used expression analysis of *H. fossilis gnrh2*, *kiss2*, *cyp19a1a*, *avt*, *it* and β -actin

Primers	Sequences (5'–3')	Amplicon size	Reference
β -actin (DNA control)	Forward: TGGCCGTGACCTGACTGAC Reverse: CCTGCTCAAAGTCAAGAGCGAC	157 bp	Chaube et al. (2015)
<i>kiss2</i>	Forward: AAGTTCCTCACGAAAAGCA Reverse: CATCCGAAGACGACAGGATT	200 bp	Chaube et al. (2020a,b)
<i>gnrh2</i>	Forward: ACAGACGAGGCAGGTGTATG Reverse: AAGGGTTCGAATCCACCATT	188 bp	Chaube et al. (2019)
<i>cyp19a1a</i>	Forward: CCATTGGCTGTGGGCCAC Reverse: ATGGTGCTGACGGTGCAACC	128 bp	Chaube et al. (2015)
<i>Vt</i>	Forward: TGTTACATCCAGAACTGCCCCAGA Reverse: CAGCCAGTCCTTCTCCACAGCA	137 bp	Banerjee et al. (2015)
<i>it</i>	Forward: TCAATCTTCTGCATGCTGTGTCT Reverse: CACACCCATGCACACTGTCTATTG	118 bp	Banerjee et al. (2015)

Green qPCR master mix with ROX (Affymetrix, Inc. Cleveland, Ohio, USA) in an ABI Prism 7500 thermal cycler (Applied Biosystems, Foster, CA, USA) at 95 °C (15 s) and 60 °C (1 min) for 40 cycles. Each sample was run in a final volume of 20 µL containing 1 µL of cDNA, 10 pM of specific primers, and 10 µL of SYBR Green PCR master mix. The primer specificity was confirmed by dissociation curve analysis. As controls, the assays were performed without templates and reverse transcriptase. No amplification was observed in the control samples. Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and the target gene expression was normalized against catfish β-actin expression to generate $2^{-\Delta\Delta C_t}$ values to quantify the target gene abundance (Livak and Schmittgen 2001). The catfish β-actin expression was stable for this species in the earlier validation study (Chaube et al. 2017).

Statistical analysis

Data are presented as the mean ± SEM. One-way ANOVA determined the significance of differences between control and treatments with Bonferroni multiple-comparison test using GraphPad Prism 9.2 software (GraphPad Software).

Results

Production of recombinant gonadotropins

Following the selection of the super clones that showed the highest expression, the large-scale production of (1 L) of rhfFSHβ and rhfLHβ was performed. The yield of the gonadotropins varied between 326 µg/L to 621 µg/L (Fig. 1B). Western blot analysis revealed that histidine tagged rhfFSHβ reacted with anti-His antibody, rhfFSHβ yielded no clear bands and a smear, probably due to excessive glycosylation, and for rhfLHβ, bands of 25 to 55 kDa were detected (Fig. 1C and D). After deglycosylation with PNGase F to reduce the excessive glycosylation, rhfFSHβ yielded a band of ~25 kDa (blacked box, Fig. 1C) and rhfLHβ yielded three bands (Fig. 1D, blacked box); the first, and strongest band, of ~27 kDa, represented the tethered rhfLHβ, while the other two bands at 15 to 22 kDa emerged, most likely, as a result of either degradation or

different degrees of glycosylation. Transformation with the vector alone (i.e., GS115/pPIC9K [Mut⁺]), serving as a negative control, showed no bands when immunoblotted (Data not shown).

In vitro effects of rhfLH and rhfFSH on sex steroid production

Our aim was to study the differential potency of stinging catfish gonadotropins in eliciting steroid secretion from catfish ovaries at different reproductive stages. The ovarian fragments were exposed to graded doses of recombinant FSH and LH. Mammalian hCG served as positive control. We examined the steroidogenic response of intact catfish follicles at two different stages of the reproductive season (previtellogenic i.e., preparatory and vitellogenic i.e., prespawning).

After 24 hr of incubation of ovary fragments during the preparatory phase (GSI = 2.5%), the production of E2 was significantly stimulated by rhfLH and rhfFSH in a concentration-dependent manner (Fig. 2A and B). Testosterone production during preparatory phase was decreased in a concentration-dependent manner (Fig. 2C). During the prespawning phase, rhfLH elicited a biphasic effect, low concentration showed no significant change with respect to control, while it decreased in a concentration-dependent manner with respect to hCG. Testosterone production was significantly decreased in a concentration-dependent manner in response to rhfFSH (Fig. 2D).

In vitro effects of rhfLH/rhfFSH on the gene expression pattern of avt, it, gnrh2, kiss2 and cyp19a1a

Our investigation showed rhfLH significantly increased AVT gene expression in a concentration-dependent manner during the preparatory stage, with the exception of the 31.25 ng/ml treatment that led to a lower response compared to the control treatment. In the prespawning phase, elevation in gene expression was observed only from the 125 ng/ml dose and continued in a dose-dependent manner. However, there was no significant change at lower concentrations with respect to control and hCG (Fig. 3A). On the other hand, rhfFSH upregulated AVT gene expression in a concentration-dependent manner (Fig. 3B).

rhfLH differentially regulated the expression pattern of the IT gene with respect to control

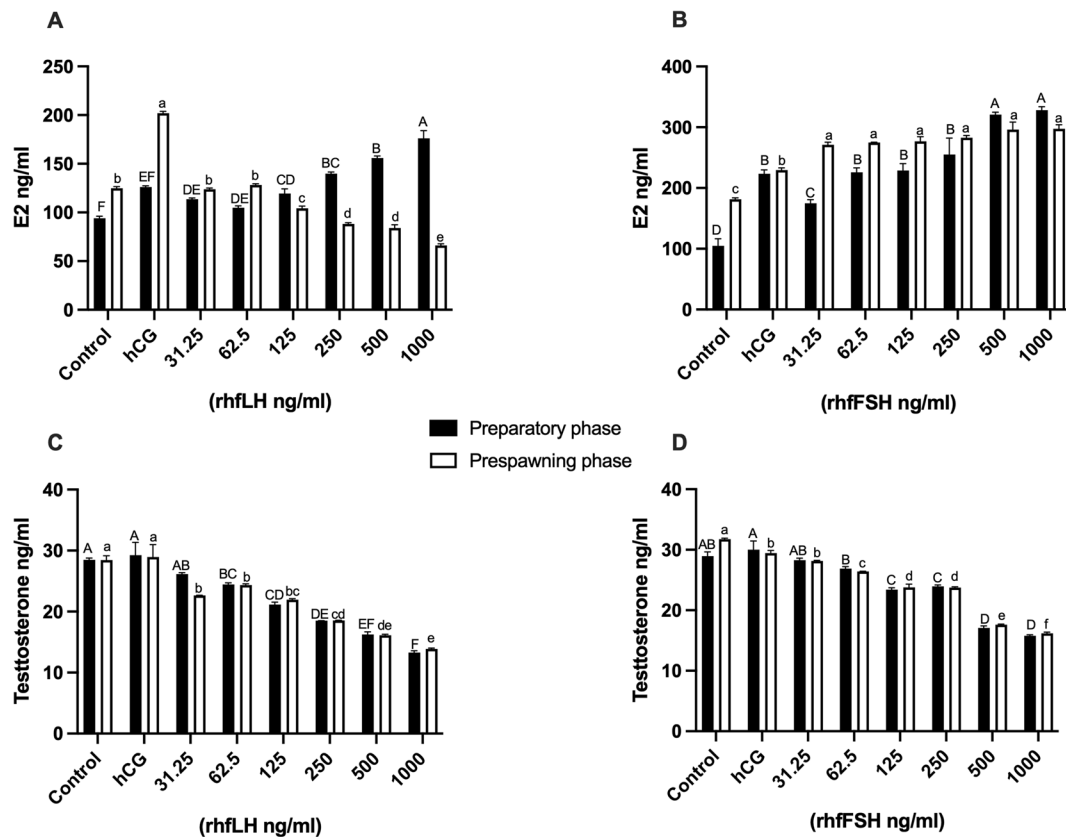


Fig. 2 In vitro effects of the different concentrations of rhfLH/rhfFSH and hCG on the production of 17 β -Estradiol (E₂) and testosterone in ovarian fragments during preparatory and prespawning phases of the reproductive cycle

during both the preparatory and prespawning phase (Fig. 3C). However, with respect to hCG it showed a biphasic effect, low dose inhibitory and high dose stimulatory. Fold change increased during the prespawning phase. rhfFSH stimulated IT gene expression in a concentration-dependent manner (Fig. 3D).

Both rhfLH and rhfFSH showed a dose response effect on *kiss2* expression gene expression in the preparatory and prespawning phase (Fig. 4A and C). The same effect was shown with GnRH2 expression pattern (Fig. 4B and D).

rhfLH had a negative effect on *cyp19a1a* gene expression in the low doses and no effect in the high doses in the preparatory phase (Fig. 5A). In the prespawning phase, only hCG and the high doses (500, 1000 ng/ml) had a positive effect on the expression of *cyp19a1a*. rhfFSH had a dose response effect on *cyp19a1a* expression in both phases (Fig. 5B).

Discussion

In this study, recombinant stinging catfish FSH and LH were produced by the *P. pastoris* protein expression system, and their bioactivities were studied and compared via in vitro experiments.

Both rhfLH and rhfFSH were produced with the commonly used single-chain linker design (Aizen et al. 2007b; Yom-Din et al. 2016; Aizen et al. 2017). Western blot analysis of the deglycosylated rhfFSH, using anti-histidine tag, showed the expected 24.4 kDa band indicating that the hormone was successfully synthesized, secreted, and harvested using the Ni-NTA beads. Western blot analysis also revealed a ~37 kDa band representing the deglycosylation reagent PNGase F. The deglycosylated rhfLH revealed 3 bands, the first, and strongest band, of ~27 kDa, represents the tethered rhfLH β α expected size, while the other two bands

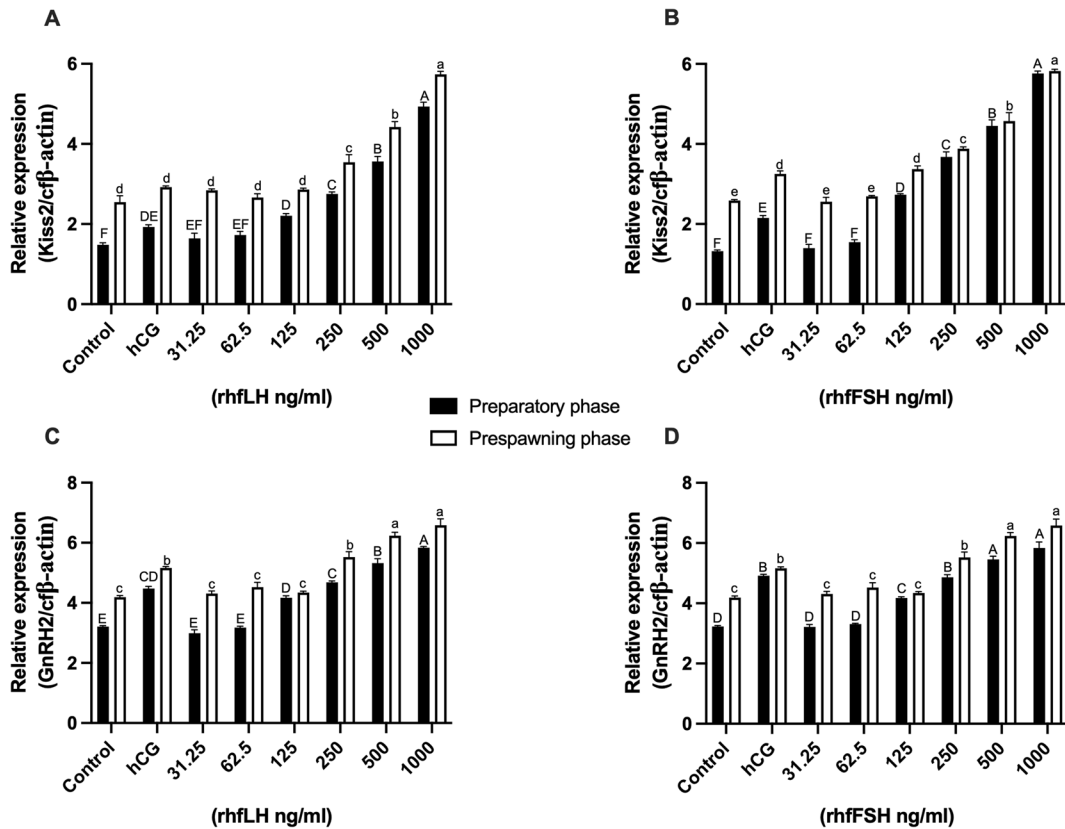


Fig. 3 *In vitro* effects of the different concentrations of rhfLH/rhfFSH and hCG on the expression pattern of *avt* and *it* in ovarian fragments during preparatory and prespawning phases of the reproductive cycle

represent either degradation or different degrees of glycosylation, as described for several recombinant fish gonadotropin productions (Kasuto and Levavi-Sivan 2005; Aizen et al. 2007b; Yom-Din et al. 2016; Aizen et al. 2017). Overall, the purification procedure yielded 326 μg of pure rhfFSH β and 621 μg of pure rhfLH β from 1 L of medium. The yields in this study are lower than other reported gonadotropins expressed in the yeast (Aizen et al. 2007b) and using other expression systems such as baculovirus for channel catfish FSH yielded 8 mg/L and for LH 6 mg/L (Zmora et al. 2003, 2007). African catfish Gths were produced in the soil amoeba *Dictyostelium discoideum* where the production levels were even lower, yielding 18–61 μg rcfFSH and 31–80 μg rcfLH per L of medium (Vischer et al., 2003). It should be taken into account that the method used was production in a flask, and using a fermenter could upscale the production by 20 fold (Aizen et al. 2017).

Recombinant FSH and LH have been successful in inducing steroid secretion in various species both in-vivo and in-vitro. E2 levels were elevated in ovaries of sea bass, yellowtail kingfish, and tiger grouper in response to rFSH and rLH (Molés et al. 2020). Testosterone showed an elevation in European eel and sole testis in response to both recombinant FSH and LH (Chauvigné et al. 2012; Peñaranda et al. 2018). The same elevation was recorded in grouper ovaries in response to recombinant FSH (Chen et al. 2012).

To verify the biological activity of the rec-GtHs in their natural target tissue, their ability to induce testosterone and estradiol secretion and the gene expression of *avt*, *it*, *gnrh2*, *kiss2*, and *cyp19a1a* was examined using the stinging catfish ovarian follicles in the preparatory and prespawning phase.

The present study shows that ovarian *avt*, *it*, *gnrh2*, *kiss2*, and *cyp19a1a* expression was differentially modulated by rec-GtHs (rhfLH and rhfFSH)

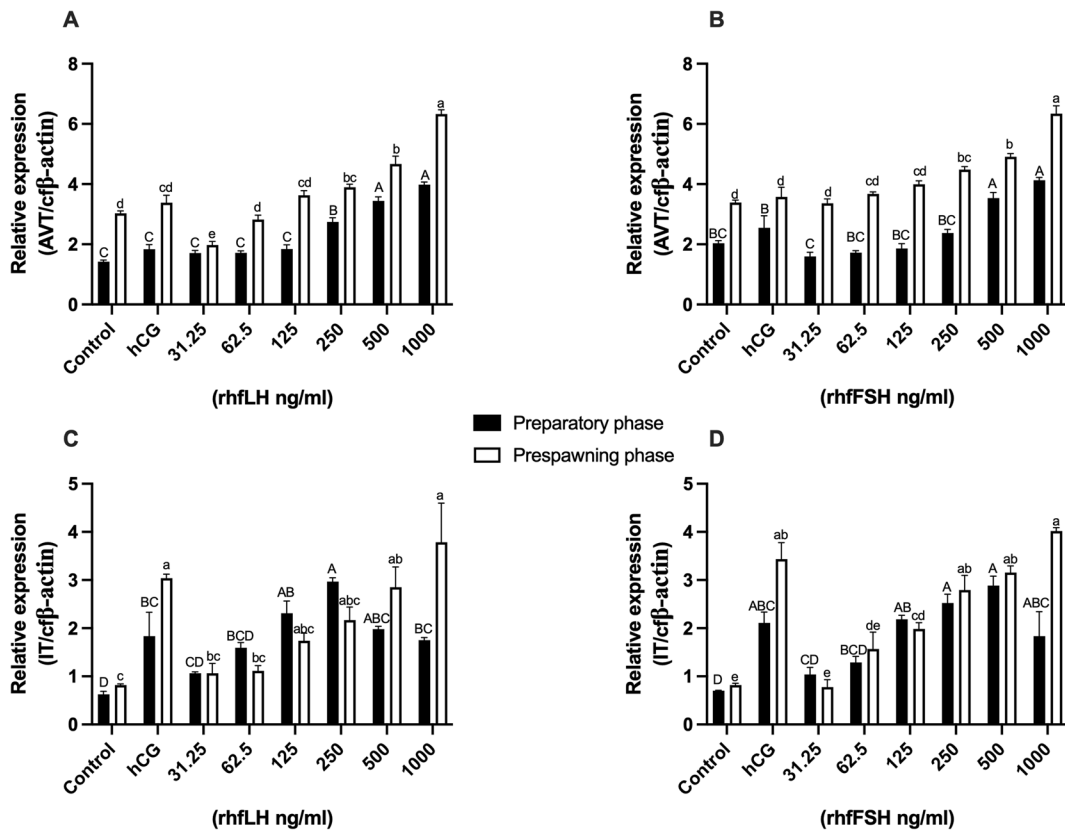


Fig. 4 In vitro effects of the different concentrations of rhfLH/rhfFSH and hCG on the expression pattern of *gnrh2* and *kiss2* in ovarian fragments during preparatory and prespawning phases of the reproductive cycle

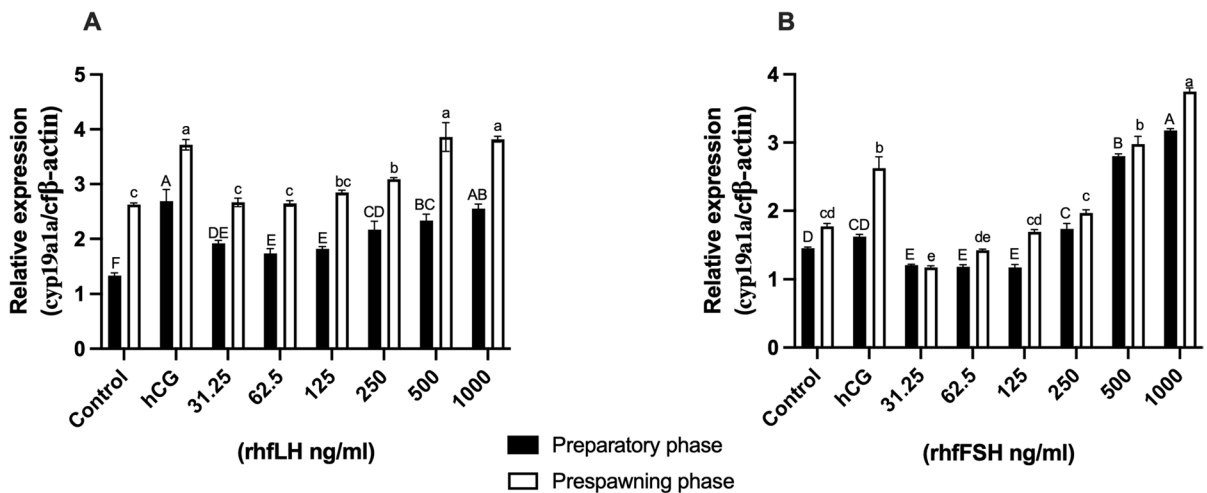


Fig. 5 In vitro effects of the different concentrations of rhfLH/rhfFSH and hCG on the expression pattern of *cyp19a1a* in ovarian fragments during preparatory and prespawning phases of the reproductive cycle

treatments under in-vitro conditions in a season and concentration dependent manner.

In the stinging catfish, *kiss2* is expressed in the ovary, localized in the follicular layer, and is associated with its development (Chaube et al. 2020a). The presence of the GnRH and Kiss systems in the ovary of the stinging catfish strongly suggest functional interactions between the two systems, as in the hypothalamus and pituitary as previously described in teleost (Gopurappilly et al. 2013; Vissio et al. 2021).

Zhang et al. (2018) reported that in the spotted scat, *Scatophagus argus*, recombinant FSH and LH significantly increased plasma concentration of steroid hormones E2 in females and 11-KT in males. mRNA expression levels of *Star*, *SaGtH*, and *17 β -hsd* were elevated, but the *Amh* expression was decreased in males or females after the injection (Zhang et al. 2018). In *S. argus*, these results indicate that recombinant LH and FSH can activate sexual steroid signaling and regulate expression of *Star*, *SaGtH*, *Amh*, and *17 β -hsd*. Similarly, in the present study we showed that rhfLH can stimulate E2 in a concentration dependent manner during the preparatory phase and decreased E2 level during prespawning phase. rhfFSH stimulated E2 levels in both preparatory and prespawning phases in a concentration dependent manner. Testosterone level was significantly decreased ($P < 0.05$) in both the preparatory and prespawning phases after incubation with rhfLH and rhfFSH under in vitro conditions.

In catfish *H. fossilis*, under in vitro conditions, the *kiss2* peptides were effective in elevating *gnrh2* expression in the ovary using high doses (Chaube et al. 2022). Previous studies in teleost have documented varied responses of Kiss peptides on GtH dynamics. Li et al. reported that ip administration of goldfish Kiss 1–10 in sexually mature female goldfish stimulated serum LH levels, but failed to influence LH release from the pituitary cells in vitro (Li et al. 2009). According to our research, elevated levels of FSH and LH are associated with increased expression of both *kiss2* and *gnrh2*. This finding may elucidate a feedback loop between the gonadotropins and the Kiss-GnRH system in the catfish in relation to ovarian development. The development of ELISA assays to measure gonadotropins levels during ovarian development could aid in further understanding this mechanism. Kim et al. determined that cinnamon clownfish rFSH and rLH increased gene expression levels

of GtH subunits, GtH receptors, and Vtg in in vitro studies as well as plasma E2 levels in immature fish. These findings support the hypothesis that LH and FSH stimulate reproduction, leading to upregulation of GtH receptors and GtH hormone production (Kim et al. 2014). Both rhfLH- and rhfFSH-induced mRNA expression of *kiss2*, *gnrh2*, *avt*, *it*, and *cyp19a1a* in a concentration dependent manner. A biphasic effect was observed whereby low doses (31.25 and 62.5 ng/ml) were inhibitory or not significant and higher doses (125, 250, 500, and 1000 ng/ml) enhanced gene expression levels significantly during the preparatory and pre-spawning phases. In catfish, hypothalamic aromatase activity is increased during the preparatory period, along with an increase in ovarian weights. The increase in enzyme activity continues up to the spawning period, then declines immediately after spawning. *cyp19a1a*, *kiss2*, *gnrh2*, *avt*, and *it* are important factors in the reproductive endocrinology of teleost through brain-pituitary-gonad-axis (Kazeto and Trant 2005). Kisspeptin is considered an upstream regulator of gonads (Chaube et al. 2020a, 2020b). In catfish *H. fossilis* the expression of the *gnrh2* mRNA in the brain-pituitary-gonadal-axis and its regulation by the gonadal steroids suggest that *gnrh2* may have a reproductive role in the catfish. The seasonal activity patterns and gonadal distribution of VT indicate a reproductive function of the peptide (Singh and Joy 2009b, 2009a). In *H. fossilis*, IT genes were expressed only in the brain and ovary (Banerjee et al. 2015). Bobe et al. 2006 reported VT and IT mRNAs in the preovulatory ovaries of rainbow trout (Bobe et al. 2006). In the present study, rhfFSH and rhfLH differentially modulated the expression pattern of *cyp19a1a*, *kiss2*, *gnrh2*, *avt*, and *it* in a season- and stage-dependent manner suggesting their role in regulating catfish reproduction. Further investigation is required at the level of oocyte maturation and ovulation.

Thus, in the present study we demonstrated for the first time that the yeast *P. pastoris* system could produce biologically active recombinant of catfish *H. fossilis* GtHs and suggests that these rGtHs could be applied to aquaculture as a substitute to a pituitary extract treatment, if a method of large-scale production is developed. Since these recombinant FSH and LH are not contaminated by each other, unlike pituitary extract, the differential use of rhfFSH and rhfLH might be able to establish a more efficient method for

the induction of gonadal development in the stinging catfish. However, further characterization and bioassay of these recombinant GtHs will be required before it can be applied to aquaculture.

This study enhances our understanding of the mechanisms of GTH-mediated control of reproduction in the catfish via the involvement of *avt*, *it*, *gnrh2*, *kiss2*, and *cyp19a1a* genes. The recombinant hfGtHs can be utilized in the artificial induction of gonadal maturation in catfish *H. fossilis* and other teleosts.

Abbreviations *E2*: 17 β -Estradiol; *T*: Testosterone; *rhfLH*: Recombinant catfish luteinizing hormone; *rhfFSH*: Recombinant catfish follicle stimulating hormone

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Author contribution JA, RC, and AE designed and coordinated the study. JA, RC, and SS performed all experiments. JA and RC wrote the manuscript. All authors (JA, SS, KPJ, AE, and RC) reviewed, finalized, and edited the manuscript and approved the final version of the manuscript.

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Data availability All of the material (figures, images, and supplementary material) is owned by the authors and/or no permissions are required. Data will be available upon request.

Declarations

Ethics approval and consent to participate All experimental procedures comply with the animal care and use guidelines of the Banaras Hindu University for experimentation of animals, and all care (permit no: 1802/GO/RE/S/15/CPCSEA) was taken to prevent cruelty of any kind.

Competing interests The authors declare no competing interests.

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