General and Comparative Endocrinology 280 (2019) 134–146

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Molecular cloning and characterization of a gonadotropin-releasing hormone 2 precursor cDNA in the catfish *Heteropneustes fossilis*: Expression profile and regulation by ovarian steroids

R. Chaube[a], A. Rawat[a], S. Sharma[a], B. Senthilkumaran[b], S.G. Bhat[c], K.P. Joy[c,*]

[a] Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005, India
[b] Department of Animal Biology, School of Life Sciences, University of Hyderabad, Telangana, Hyderabad 500046, India
[c] Department of Biotechnology, Cochin University of Science and Technology, Kochi 682022, India

ARTICLE INFO

Keywords:
Catfish
Gnrh2 precursor cDNA
Cloning
Phylogeny
Steroid regulation
Tissue and seasonal expression

ABSTRACT

Gonadotropin-releasing hormone 2 (Gnrh2) is one of the three classes of Gnrh distributed in vertebrates and is highly conserved. In the present study, the cDNA encoding Gnrh2 was isolated and characterized in the ostariophysan catfish *Heteropneustes fossilis (hf)*. The cDNA is 611 bp long with an open reading frame (ORF) of 261 bp that encodes a highly conserved protein of 86 amino acids. The deduced Gnrh2 precursor protein clustered with the vertebrate Gnrh2 type. The sequence identity of *hfgnrh2* is 94% with African catfish (*Clarias gariepinus*) *gnrh2* mRNA (accession no. X78047). The *hfgnrh2* transcripts were expressed only in the brain and gonads with a higher expression in the female brain and ovary in both resting and prespawning phases. The expression was higher in the prespawning phase than the resting phase. The *gnrh2* expression in the brain and ovary showed significant seasonal variations but with opposite patterns. In the brain, the expression was the highest in the preparatory phase, decreased progressively to low levels in the postspawning and resting phases. In the ovary, the transcript level was low in the resting and preparatory phases, increased sharply in the prespawning phase reaching the peak level in the spawning phase and declined sharply in the postspawning phase. The *gnrh2* mRNA showed the highest expression in the hind brain-medulla oblongata and moderate to low expression in forebrain regions and pituitary. Ovariectomy resulted in a duration-dependent inhibition of *hfgnrh2* mRNA levels in the resting and prespawning phases. Steroid (E_2, testosterone and progesterone) replacement treatments (0.5 μg/g body weight) in the 3- week ovariectomized fish restored the inhibition due to ovariectomy, elevated the expression over and above the sham level in the resting phase (E_2 group), and raised the levels almost to that of the sham group (testosterone and progesterone groups) in the prespawning phase. In the sham control groups, the steroid replacement resulted in a significant reduction in the mRNA levels. The expression of the *gnrh2* mRNA in the brain-pituitary-gonadal axis and its regulation by gonadal steroids suggest that Gnrh2 may have a reproductive role in the catfish.

1. Introduction

The onset of puberty and reproduction are controlled by concerted and coordinated actions of regulators involving the brain-pituitary-gonadal (BPG)-endocrine axis. The BPG axis mediates and integrates the changes in environmental factors such as photoperiod, temperature, food and stress through the intervention of a plethora of chemical messengers collectively called as neurohormones (neurotransmitters and neuropeptides). The neuropeptide gonadotropin-releasing hormone (Gnrh) has been considered till recently the central molecule initiating reproductive activity in vertebrates. Gnrh stimulates pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion (Fernald and White, 1999; Chen and Fernald, 2008; Lee et al., 2008; Okubo and Nagahama, 2008; Chang and Pemberton, 2018), which stimulates tertiary regulators such as steroids, peptides and growth factors in the gonads during gametogenesis. In addition, Gnrh acts as a neuromodulator and has also been implicated in feeding and reproductive behavior in many species including teleosts (Yamamoto et al., 1997; Volkoff and Peter, 1999; Xia et al., 2014). To date, ~18 forms of Gnrhs have been identified based on their primary structure or complementary DNAs (cDNAs) in vertebrates (Roch et al. 2014; Chang and Pemberton, 2018). Multiple forms of Gnrh exist in vertebrates and

* Corresponding author.
E-mail address: kpjoybhu@gmail.com (K.P. Joy).

https://doi.org/10.1016/j.ygcen.2019.04.021
Received 7 February 2019; Received in revised form 10 April 2019; Accepted 16 April 2019
Available online 20 April 2019

are classified into the genus-specific, hypophysiotropic Gnrh1 localized in the preoptic area, the vertebrate Gnrh2 or chicken Gnrh2 in the midbrain and/or the teleost-specific Gnrh3 in the olfactory system (Fernald and White, 1999; Lethimonier et al., 2004; Karigo and Oka, 2013; Roch et al., 2014; Chang and Pemberton, 2018). Gnrh1 and Gnrh3 may be the same or distinct depending on the teleost species (Parhar, 1997; Gonzalez-Martınez et al., 2001; Vickers et al., 2004; Mohamed et al., 2005; Mohamed and Khan, 2006). Structurally, the prepro-Gnrh protein is composed of a signal peptide (SP), and Gnrh and a Gnrh-associated peptide (GAP), separated by the proteolyte cleavade site Gly-Lys-Arg (Fernald and White, 1999; Kah et al., 2007). The decapeptide Gnrh2 is conserved in all vertebrates but its functions are yet to be established (Chang and Pemberton, 2018).

Complementary DNAs encoding multiple forms of Gnrh have been isolated from a number of teleost species; the majority are from the species-rich superorder Acanthopterygii (NCBI database, see also Table 2) and only limited studies have been reported from the other species-rich superorder Ostariophysi (Torgersen et al., 2002; Steven et al., 2003). Only few catfish species belonging to this superorder were investigated for Gnrh isolation, cloning and characterization. In the Thai catfish *Clarias macrocephalus* and African catfish *C. gariepinus*, two forms of Gnrh were characterized by protein purification methods, a catfish-specific Gnrh1 and Gnrh2 (Bogerd et al., 1992; Ngamvongchon et al., 1992). The cDNAs coding for these proteins were isolated only in the African catfish (Bogerd et al., 1994). Additional studies in ostariophysan species will throw light on the diversity, evolutionary relationships and functions of the neuropeptide in Teleostei, the largest group of vertebrates.

Gonadal steroids exert negative or positive feedback control on pituitary FSH and LH secretion by interacting with brain neuropeptides and neurotransmitters. Estrogens, androgens and progesterone (P_4) have been implicated in the control of Gnrh secretion but their effects are varied at different reproductive stages (Lee et al., 2008; Brayman et al., 2012). Though previous reports have disputed the occurrence of estrogen receptors (ERs) on Gnrh neurons (see Herbison, 1998; Marques et al., 2015), studies especially in Gnrh cell lines have documented a direct involvement of estrogens in Gnrh cell activities (Radovick et al., 1991; Roy et al., 1999; Pak et al., 2006; Wolfe and Wu, 2012). The presence of an estrogen-response element in the promoter region of the human Gnrh1 gene and the presence of ERs on hypothalamic and ovarian Gnrh cell lines suggest the involvement of estrogens in Gnrh gene expression (Lee et al., 2008). Estrogens elicit suppressive or stimulatory effects on Gnrh1 and Gnrh2 gene expression depending on the reproductive stage. Androgens down regulate Gnrh mRNA levels through an androgen receptor-mediated mechanism (Brayman et al., 2012). P_4 modulates Gnrh secretion differently through a P_4 receptor mechanism; the stimulation or repression depends on the reproductive conditions in humans and animals (Cho et al., 1994; Lee et al., 2008). In teleosts, studies on the regulation of Gnrh gene expression by gonadal steroids are limited (Parhar et al., 2000; Shao et al., 2015; Alvarado et al., 2016) and the results are conflicting.

In the present study, the gene encoding prepro-Gnrh2 precursor was cloned as cDNA and characterized in *Heteropneustes fossilis*, an air-breathing catfish inhabiting freshwater bodies of the Indian subcontinent. The catfish has been extensively used as an important animal model for reproductive endocrinology research and is an emerging aquaculture species. Additionally, phylogenetic relationship, tissue expression and steroid hormone regulation of *gnrh2* expression were investigated.

2. Materials and methods

2.1. Animal collection and acclimatization

Adult male and female catfish *Heteropneustes fossilis* (Bloch) (Order: Siluriformes; Family Heteropneustidae or Saccobranchidae) of the first year sexual cycle (45–65 g) were collected from local fish markets at Varanasi in different reproductive phases: resting (January–February), preparatory (March–April), pre-spawning (May–June) and spawning (July–August) and postspawning (September–October). They were maintained in the laboratory for a fortnight under natural photoperiod and temperature conditions to overcome stress due to transportation and fed with goat liver *ad libitum*. All experiments were performed in accordance with the general guidelines of the Institutional Ethics Committee of Banaras Hindu University, Varanasi.

2.2. Chemicals and reagents

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), 2xPCR Mastermix (Fermentas, Foster, CA USA), VeriQuest™ SYBR Green qPCR Master mix with ROX (Affymetrix, Inc. Cleveland, Ohio USA), Nucleopore PCR clean up gel extraction kit (Genetix, Genetix Asia Pvt. Ltd., New Delhi), 3′ and 5′ RACE anchor primer (3′ RACE, Roche Applied Science, Penzberg, Germany), dATP (Roche Applied Science, Penzberg, Germany) and pGEM-T Easy Vector (Promega, Madison, Wisconsin USA) were obtained through local suppliers. The primers were synthesized by Integrated DNA Technologies, Hyderabad, India. Tricaine methanesulfonate (MS 222), Estradiol-17β (E_2), testosterone, progesterone (P_4), Tris base and EDTA (ethylene diamine tetraacetic acid) were purchased from Sigma-Aldrich, New Delhi, India. IPTG (isopropyl-1-thio-*b*-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-*b*-D-galactopyranoside), Agarose and other chemicals were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. ELISA kits for E_2, testosterone and P_4 (Dia Metra, Italy REF-DKO003, LOT-4511A, REF-DKO002, LOT-4510A, REF-DKO006, LOT-4720A, respectively) were purchased locally.

2.3. Total RNA isolation and cDNA synthesis

For cloning, three female fish were euthanized with an overdose of MS 222 in the prespawning phase and brains were dissected out and stored in RNAlater solution briefly. Total RNA was extracted from the whole brain using RNeasy kit and treated with 2U DNase I, RNase-free for 30 min at 37 °C, following the manufacturer's protocol. The quality of RNA was analyzed after resolving on a 2% agarose gel containing 2.2 M formaldehyde and absorbance was measured at 260 and 280 nm. The RNA samples with a 260/280 ratio between 1.8 and 2.0 were used in cDNA synthesis. cDNA was synthesized from 5 μg total RNA with random hexamer primer using Revert-Aid H minus M-MuLV reverse transcriptase, according to the manufacturer's instructions.

2.4. Amplification of a partial cDNA sequence by degenerate primer PCR

First strand cDNA was synthesized in 20 μL reaction mixture (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dNTPs, 0.5 U recombinant RNase inhibitor, 100 pM hexamer primer and 200 U M-MuLV reverse transcriptase) at 42 °C for 1 h. The reaction mixture was diluted to a 100 μL final volume by adding 80 μL of DEPC-treated water. PCR was performed using degenerate primers (Rose et al., 2003), designed from the conserved regions of cDNA sequences of fish Gnrh2 using the software CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers). The details of the degenerate primers are provided in Table 1. The degenerate primer PCR was performed in 50 μL reaction containing 12.5 μL of master mix (1xPCR buffer plus Mg2+, 0.2 mM dNTPs, 1 IU of Taq polymerase), 9.5 μL RNase/DNase-free water, 1 μL each of forward and reverse degenerate primers (25 pM) and 1 μL reverse transcribed cDNA template. The reactions were run under a thermal profile of 94 °C for 5 min, 35 cycles at 94 °C for 50 s, 55–57 °C for 1 min, and 72 °C for 1–2 min, followed by 72 °C for 7 min. The PCR

Table 1
Primers used for cloning and expression of *gnrh2* and expression of β-actin control.

Primer	Gene	Direction and Sequence (5′–3′)	Amplicon size (bp)
Degenerate	*gnrh2*	Forward (P1) ACAGGCTCTGATCAAGCARGGNGANGA Reverse (R1) CACCACGATGTGTCGGAANGGRTCRAA	298
Specific	*gnrh2*	Forward (P2) ACAGACGAGGCAGGTGTATG Reverse (R2) AAGGGGTCGAATCCACCATT	188
3′ Race	*gnrh2*	Forward (P3) ACAGACGAGGCAGGTGTATG Forward (P4) TCTGCGCTCCTTTGGTATGA Anchor primer GACCACGCGTATCGATGTCGAC	
5′Race	*gnrh2*	Reverse (R3) AAGGGGTCGAATCCACCATT Reverse (R4) TCAGGTACAGGCCCTCTTTG	
qPCR	*gnrh2*	Forward (P5) GTTCAGCACAGACGAGGCA Reverse (R5) CTGATGTGTTCCTCCAGGGCA	145
DNA control	*β-actin*	Forward (P6) TGGCCGTGACCTGACTGAC Reverse (R6) CCTGCTCAAAGTCAAGAGCGAC	157

Table 2
GenBank accession numbers of Gnrh 1, Gnrh 2 and Gnrh 3 protein sequences of different vertebrates used in phylogenetic tree construction and analysis.

Common Name	Scientific Name	GenBank accession number
Gnrh 1		
Japanese eel	*Anguilla japonica*	BAA82608.1
Giant mottled eel	*Anguilla marmorata*	ACV66343.1
European eel	*Anguilla anguilla*	BAA82608.1
Burton's mouth brooder	*Haplochromis burtoni*	NP_001273225.1
Human	*Homo sapiens*	NP_000816.4
Nile tilapia	*Oreochromis niloticus*	AAM90220.1
Lake whitefish	*Coregonus clupeaformis*	AAP57219.1
House mouse	*Mus musculus*	AAI16900.1
Walking catfish	*Clarias batrachus*	AIU98448.1
Japanese rice fish	*Oryzias latipes*	NP_001098169.1
Common leopard gecko	*Eublepharis macularius*	ABB89899.1
Green Spotted Puffer	*Tetraodon nigroviridis*	BAE45688.1
Flathead grey mullet	*Mugil cephalus*	AAQ83269.1
Chicken	*Gallus gallus*	NP_001074346.1
Black salmon	*Rachycentron canadum*	AAT80334.1
African clawed frog	*Xenopus laevis*	XP_018107225.1
Asian swamp eel	*Monopterus albus*	AAW51121.1
Gnrh 2		
Tropical clawed frog	*Xenopus tropicalis*	NP_001107165.1
Zebrafish	*Danio rerio*	NP_852104.3
Lake whitefish	*Coregonus clupeaformis*	AAP57219.1
Channel catfish	*Ictalurus punctatus*	XP_017348636.1
Common carp	*Cyprinus carpio*	XP_018951985.1
European eel	*Anguilla anguilla*	ADD92005.1
Japanese eel	*Anguilla japonica*	BAA82609.1
African sharptooth catfish	*Clarias gariepinus*	P43306.1
Goldfish	*Carassius auratus*	XP_026052200.1
Japanese rice fish	*Oryzias latipes*	Q9DGC9.1
Green Spotted Puffer	*Tetraodon nigroviridis*	BAE45690.1
Atlantic cod	*Gadus morhua*	ADD92006.1
Asian swamp eel	*Monopterus albus*	AAV41875.1
Flathead grey mullet	*Mugil cephalus*	AAQ83270.1
Leopard gecko	*Eublepharis macularius*	BAC99084.1
Rainbow trout	*Oncorhynchus mykiss*	NP_001117717.1
Gnrh 3		
Gold fish	*Carassius auratus*	XP_026142592.1
Common carp	*Cyprinus carpio*	XP_018926565.1
Atlantic cod	*Gadus morhua*	ADD92007.1
Atlantic salmon	*Salmo salar*	NP_001117139.1
Lake whitefish	*Coregonus clupeaformis*	AAP57220.1
Green Spotted Puffer	*Tetraodon nigroviridis*	BAE45692.1
Asian swamp eel	*Monopterus albus*	AAW51120.1
Nile tilapia	*Oreochromis niloticus*	AAO11648.1
Black salmon	*Rachycentron canadum*	AAT80332.1

products were separated on 2% agarose gel and the reaction products containing bands of expected sizes were extracted by Nucleopore PCR clean up gel extraction kit. Sequencing was done by taking the service of Eurofins Genomics, Bengaluru, India.

2.5. 3′ and 5′ rapid amplification of cDNA ends (RACE)

RACE was used to generate 3′ and 5′ ends of *gnrh2*, designed from the partial sequence. The 3′ RACE product was amplified by PCR using primer P3 and the 3′ RACE anchor primer (Roche Applied Science) with 20 ng cDNA as the template (Table 1). Nested PCR was carried out with primer P4 and the PCR anchor primer to increase specificity and intensity. The 5′ RACE was carried out according to the procedure described by Frohman et al. (1988). Five micrograms of total RNA was reverse transcribed using primer R3 and with M-MuLV reverse transcriptase. The reaction product was purified using the DNA extraction kit and then appended with poly (A) using dATP (Roche Applied Science) and purified again. The 5′ RACE–PCR was then carried out using the above dA-cDNA as the template and primer R4 and oligo (dT) anchor primer in a procedure similar to the 3′ RACE.

2.6. Cloning and sequencing of gnrh2 cDNA

The purified PCR products were ligated into pGEM-T easy plasmid vector and transformed into *Escherichia coli* competent cells DH5α. The transformed cells were plated on ampicillin – agar medium with isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indlyl-β-D-galactopyranoside (X-gal) for blue/white color screening. White colonies were selected from X-Gal/IPTG ampicillin – agar plates and grown in LB/ampicillin liquid medium. Plasmids were extracted by the alkaline lysis method. Sequencing was done by taking the services of Eurofins Genomics. The sequences were analyzed with the BLAST-N 2.0 search tool provided by NCBI.2.4. The putative signal peptide of Gnrh2 precursor was predicted using the SignalP4.1 Server. Sequence alignment was performed using Clustal Omega and was modified manually.

2.7. Phylogenetic analysis

The deduced amino acid sequences of catfish Gnrh2, were aligned by Clustal W, version 1.7 (Thompson et al., 1994), using default settings (gap opening penalty 10, gap extension penalty 0.05, gap distance 8). MEGA version 6 (Tamura et al., 2013) software was employed to construct the phylogenetic tree using the maximum likelihood method based on the JTT matrix –based model (Jones et al., 1992). All the protein sequences were obtained from GenBank. The accession numbers, scientific names and common names are given in Table 2.

2.8. Tissue, sex dimorphic, brain regional and seasonal expressions of gnrh2

For tissue and sex dimorphic expression studies, brain, ovary, testis, gill, kidney, liver and muscle were sampled from five male and 5 female catfish each in the resting and prespawning phases (sample size = 5 fish for each phase). For seasonal study, brain and ovary were sampled from

female catfish in resting, preparatory, prespawning, spawning and post spawning phases (sample size = 5 fish for each phase). For brain region study, brains from the prespawning catfish were only used. Brains were dissected out and divided into olfactory bulb (Ob), telencephalon (Tel), hypothalamus (Hyp), thalamus (Th), cerebellum (Cb), Hindbrain + medulla oblongata (Hb). For brain region sampling, 5 fish brains were used to make each sample (total pooled sample size = 5). For pituitary sampling, pituitaries from 15 fish were pooled to make one sample and 5 such pooled samples were used (total pooled sample size = 5).

About 100 mg of each tissue was used for the extraction of total RNA by using the RNeasy mini kit (Qiagen), as described above. RNA purity was checked by calculating A260/A280 ratio. Samples having a ratio of 1.8 to 2.0 were only used. Absence of genomic DNA contamination in RNA was confirmed by using non-reverse transcribed samples as templates. In addition, the absence of DNA in total RNA was ensured by treating with DNAse I before proceeding for the first strand cDNA synthesis. Five μg of total RNA was reverse transcribed using random hexamer primers and Revert Aid M-MuLV reverse transcriptase in a 20 μL reaction volume (first strand cDNA synthesis kit, Fermentas), using the manufacturer's protocol. The primer specificity was confirmed by dissociation curve analysis. Quantitative PCR assays were performed in triplicate for different samples using specific primers (P5 and R5) and VeriQuest TM SYBR Green qPCR master mix with ROX (Affymetrix, Inc. Cleveland, Ohio USA) in a ABI Prism 7500 thermal cycler (Applied Biosystems, Foster, CA, USA) at 95 °C (15 s), 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 each primer, and 10 μL of SYBR Green PCR master mix. Specificity of amplicons was verified by melting curve analysis (60–95 °C) after 40 PCR cycles. As controls, the assays were performed without the template and reverse tarnscriptase. No amplification was observed in the control studies. Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and target gene (*gnrh2*) expression was normalized against the *β-actin* gene expression to generate $2^{-\Delta\Delta Ct}$ values to quantify the target gene abundance (Livak and Schmittgen, 2001). As an internal standard, catfish *β-actin* was used in the qPCR assay and its expression was stable in the validation studies in the catfish. The relative expression stability of *β-actin, eEF-1α* and *gapdh* was checked using the statistical programs, NormFinder and geNorm. All of them showed M values lower than 0.5 and hence can be used as potential reference genes for qPCR assays (Chaube et al., 2017).

2.9. Effects of ovariectomy (OVX) on brain gnrh2 expression

OVX was conducted in the resting and prespawning phases. The acclimated fish were anaesthetized by spraying MS222 (100 mg/250 mL distilled water) over the gills. Approximately a 3–4 cm long midventral incision was made anterior to the urogenital pore with a sterilized scalpel to expose the paired ovary. The ovaries were carefully detached from the peritoneal covering and removed. The cut end of the oviduct was cauterised with a hot needle to prevent regeneration and the incision was sutured. The fish were treated with benzanthine penicillin (16000 IU/L of water) for 3–5 days to prevent skin infection. For sham OVX, all the above steps were followed except the removal of ovary. The operated fish were maintained for 1, 2, 3 and 4 weeks. For sampling, the fish were anesthetized by spraying MS222 over the gills. Blood was collected by caudal puncture by using heparinized syringes for separation of plasma for steroid assays. The fish were sacrificed by decapitation, brains were dissected out immediately, transferred to RNAlater and stored at −80 °C. Quantitative PCR assays were conducted as described above.

2.10. Effects of E_2, testosterone and P_4 on brain gnrh2 expression

Three-week ovariectomized and sham-ovariectomized fish in both the resting and preparatory phases (12 groups each, (n = 5 fish each) were injected with E_2, testosterone or P_4 intraperitoneally in a dosage of 0.5 μg/g body mass. As controls, ovariectomized and sham-ovariectomized fish (10 groups, n = 5 each) were given an equal volume (0.1 mL) of vehicle (propylene glycol). After 24 h of the single injection, the fish were anesthetized by spraying MS222 over the gills. The fish were sacrificed by decapitation, brains were dissected out immediately and transferred to RNAlater and stored at −80 °C. Quantitative PCR assays were conducted as described above.

2.11. Steroid assay

The stored plasma samples of fish from different groups were extracted with diethylether for the steroid assay. Each plasma sample was extracted thrice with diethylether and the ether fractions were collected and pooled to make one sample (n = 5 per group). The ether fraction was evaporated under nitrogen and stored at −20 °C until the assay. Specific ELISA kits for E_2, testosterone and P_4 (Dia Metra, Italy) were used for the assay, according to the manufacturer's procedure (Singh and Joy, 2009; Chaube and Mishra, 2012). The sample volumes used were 20 μL for E_2 and 50 μL each for testosterone and P_4, as per the assay kits procedure. The response–concentration curves for E_2 were linear over 20–3200 pg/mL. The sensitivity of the assay was 10 pg/mL. Cross reactivity of the E_2 antibody was: estradiol-17*β*, 100%; estriol, 1·6%; estrone, 1·3%; progesterone, 0·1%; cortisol, 0·1%; testosterone, 0%; deoxycorticosterone, 0%; 17, 20*β*-dihydroxyprogesterone (DP) 0%. The inter- and intra-assay variations were 9·18 and 9·4%, respectively. The response–concentration curves for testosterone were linear over 0·1–20 ng/mL range. The sensitivity of the assay was 0·022 ng/mL. Cross reactivity of the testosterone antibody was: testosterone, 100%; 5*α*- dihydrotestosterone, 5·2%; androstenedione, 1·4%; androstanediol, 0·8%; progesterone, 0·5%; androsterone, 0·1%; E_2 0·001%; 17, 20*β*-DP, 0%. The inter- and intra-assay variations were 7·1 and 6.8%, respectively. The response–concentration curves for P_4 were linear over 0·1–60 ng/mL. The sensitivity of the assay was 0·1 ng/mL. Cross reactivity of the P_4 antibody was: P_4, 100%; 17-OHP$_4$, 0·4%; deoxycorticosterone, 1·7%; 17, 20*β*-DP, 4·6%; E_2, 0%; testosterone, 0·0004%; corticosterone, 0·3%; pregnenolone, 0·2%; 5-*α*- androstan-3*β*, 17*β*-diol, 0·3%; cortisol, 0%. The inter- and intra-assay variations were 10·4 and 11·4%, respectively.

2.12. Statistical analysis

Gene expression data are presented as mean of Log RQ ± SEM. The control sample was taken as the calibrator to give a relative ratio of the mRNA expression. All data were checked for homogeneity and normality and further analyzed for statistical significance by one way or two way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences software program (version 10.0; SPSS). Multiple group comparisons were made by Newman-Keuls' test ($p < 0.05$). Steroid data were presented as mean ± SEM (N = 5) and analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls' test ($p < 0.05$).

3. Results

3.1. Cloning of gnrh2 cDNA

Using the degenerate primer PCR, a 150 bp partial cDNA was isolated (Supplementary file, Fig. 1), which was used for amplification by RACE to clone the full length *gnrh2* cDNA. The cDNA is 611 bp long containing an open reading frame (ORF) of 261 bp between the start codon (nt 73) and stop codon (nt 331), a 72 bp 5′ untranslated region (UTR) and a 278 bp 3′ UTR (Supplementary file, Fig. 2). A polyadenylation signal (AATAAA) is also present at the 3′ UTR. The ORF encodes a putative protein of 86 amino acids (aa) (Supplementary file,

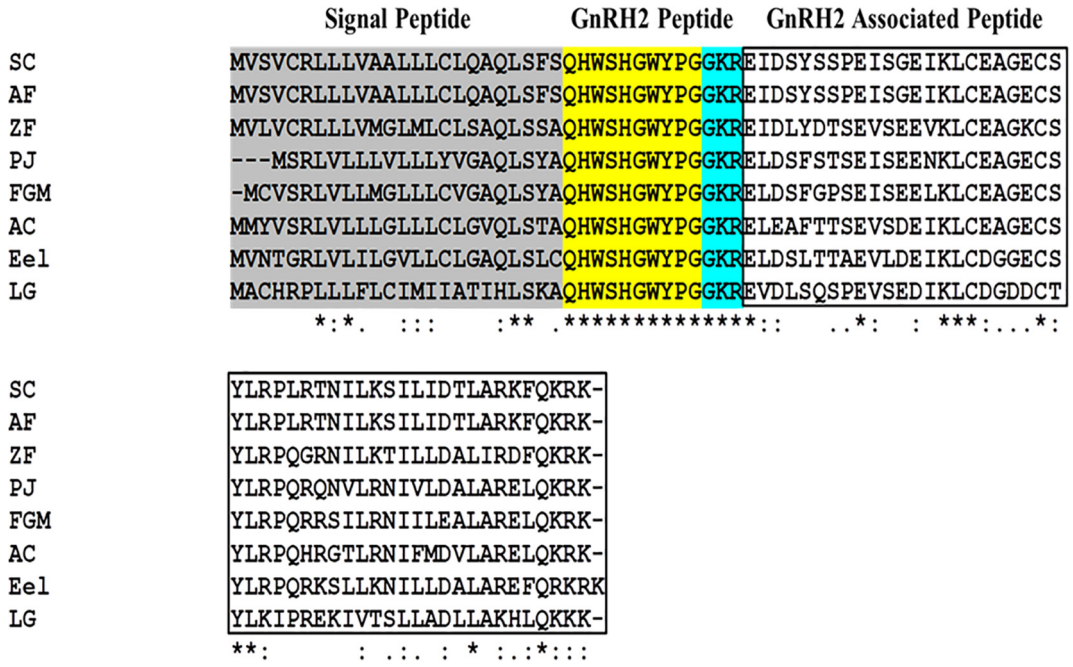

Fig. 1. Alignment of *Heteropneustes fossilis* prepro-gonadotropin–releasing hormone 2 (Gnrh2) amino acid sequence with corresponding sequences from Eel (*Anguilla anguilla*, ADD92005), African catfish, AF (*Clarias gariepinus*, CAA54969), Pejerrey, PJ (*Odontesthes bonariensis*, AAU94307), Zebrafish, ZF (*Danio rerio*, AAU43784), Atlantic cod, AC (*Gadus morhua*, ADD92006), Leopard gecko, LG (*Eublepharis macularius*, BAC99084), Flathead gray mullet, FGM (*Mugil cephalus*, AAQ83270), SC (stinging catfish, *Heteropneustes fossilis*). The four domains of the deduced protein *viz*., signal peptide (gray), Gnrh2 (hormone, yellow), proteolytic cleavage site (blue) and Gnrh2-associated peptide (rectangular block), are conserved in all species.

Fig. 2). The predicted protein showed the highest sequence identity of 94% with the African catfish (*C. gariepinus*) Gnrh2 precursor protein (accession no. X78047). The sequence was submitted to NCBI (accession number MF166829.1).

3.2. Structure of deduced Gnrh2 precursor protein

The H. fossilis Gnrh2 protein when aligned with known teleost sequences of Gnrh2 from the NCBI database showed conserved domains (Fig. 1). The catfish Gnrh2 is composed of a signal peptide (SP) of 24 aa, Gnrh2 peptide of 10 aa and a Gnrh2-associated peptide (GAP) of 49 aa. The hormone moiety is separated from the GAP by the cleavage site GKR sequence.

3.3. Phylogenetic analysis

A phylogenetic tree was constructed using the protein sequences of Gnrh of selected species obtained from the database (Table 2; Fig. 2). In the phylogenetic tree, the vertebrate Gnrh proteins clustered into three clades, namely Gnrh1, Gnrh2 and Gnrh3. The *H. fossilis* Gnrh2 protein was aligned closely with *C. gariepinus* Gnrh2 and clustered with the Gnrh2 type in the Ostariophysan subclade, away from the Acanthopterygian subclade, of teleosts.

3.4. Tissue and sex dimorphic expression of gnrh2

In *H. fossilis*, the *gnrh2* transcript was expressed highly in the brain, followed by gonads. The expression was low in the pituitary and not detected in other tissues like liver, muscle, kidney and gills (Fig. 3; $p < 0.001$, one way ANOVA, $F = 149.87$). In the gonads, the expression was higher in the ovary than testis. A two way ANOVA showed significant interactions ($p < 0.01$) between season versus brain ($F = 17.04$) and season versus gonads ($F = 14.06$).

3.5. Regional expression of gnrh2 in the brain and pituitary

The *gnrh2* transcripts were expressed in all regions of the brain investigated and the regional expression showed differences between seasons (Fig. 4; $p < 0.001$, one way ANOVA, $F = 199.12$). A two way ANOVA of the data showed overall significant sex differences in the brain regions in the resting ($F_{male} = 12.17$; $F_{female} = 15.12$ $F_{male\ x\ female} = 8.65$; $p < 0.01$) and prespawning ($F_{male} = 16.23$; $F_{female} = 18.02$ $F_{male \times female} = 10.15$, $p < 0.01$) phases. The expression was the highest in the hind brain-medulla oblongata in both sexes ($p < 0.05$, Newman-Keuls' test). In the forebrain regions, olfactory bulb, telencephalon and hypothalamus showed significant seasonal differences. In the thalamus, the expression varied with a low level in the prespawning phase in both sexes. The expression in the cerebellum was not significantly different in males between seasons, but in females a decrease was noticed in the prespawning phase. The pituitary *gnrh2* expression increased significantly in the prespawning phase with higher levels in females ($p < 0.05$, Newman-Keuls' test).

3.6. Seasonal expression of gnrh2

Noticing the differences in the *gnrh2* expression levels between resting and prespawning phases (Fig. 3), a detailed seasonal expression study was conducted in different reproductive phases of the female catfish. The transcript abundance of *gnrh2* showed significant seasonal variations in both brain and ovary (Fig. 5; $p < 0.001$, one way ANOVA, $F = 114.16$). In the brain, the expression level was high in the resting phase, unlike the ovary, increased to the peak in the preparatory phase and decreased in the prespawning and spawning phases to reach the lowest level in the postspawning phase. In the ovary, the expression level was low in the resting and preparatory phases and increased sharply in the prespawning phase to the peak level in the spawning phase. After the spawning phase, the expression decreased to low levels in the post spawning phase.

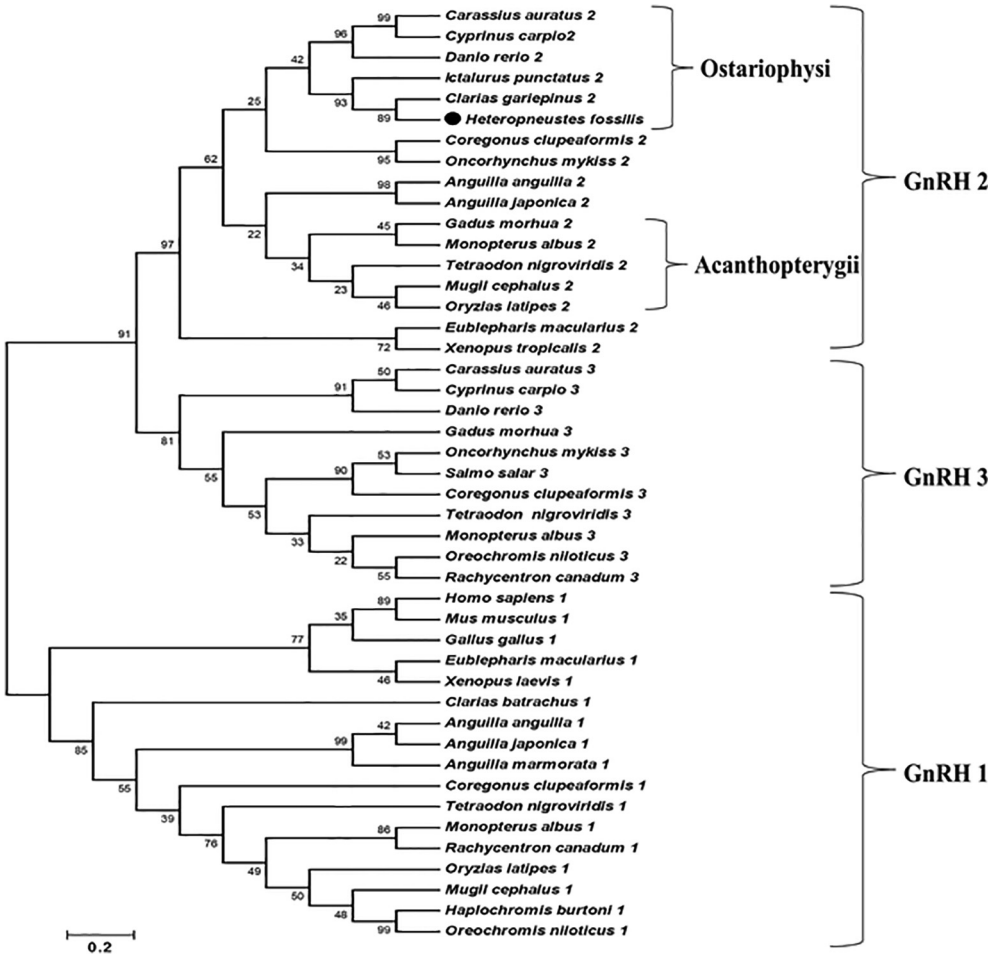

Fig. 2. Phylogenetic analysis of catfish (*Heteropneustes fossilis*) Gnrh2. Phylogenetic analyses were conducted in MEGA6 using the Maximum-Likelihood method and were bootstrapped (1000 replicates) to assess robustness. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is illustrated next to the branches. The branch lengths were used to define evolutionary distances. In the tree, the catfish Gnrh2 aligned closely with the African catfish (*C. gariepinus*) Gnrh2 in the ostariophysan subclade.

3.7. Effects of OVX on plasma steroid levels

OVX caused overall significant effects on plasma E_2 level (Supplementary file, Fig. 3; $p < 0.001$, one way ANOVA) in resting ($F = 82.13$) and prespawning ($F = 25.63$) phases. The E_2 levels were decreased significantly over the time of OVX ($p < 0.05$, Newman-Keuls' test). In the sham groups, plasma E_2 levels increased gradually over the time. Plasma testosterone level decreased significantly after OVX in the resting and prespawning phases (Supplementary file, Fig. 4; $p < 0.001$, one way ANOVA; resting phase $F = 54.08$, prespawning phase, $F = 32.17$) compared to the respective sham groups ($p < 0.05$, Newman-Keuls' test). While the testosterone levels decreased in a duration-related manner in the resting phase, the decrease was not time-dependent in the prespawning phase. In the resting phase, plasma P_4 level decreased significantly after OVX in a duration-dependent manner (Supplementary file, Fig. 5; $p < 0.001$, one way ANOVA; $F = 47.12$; $p < 0.05$, Newman-Keuls' test). In the prespawning phase, plasma P_4 level did not change significantly in the first week of OVX but decreased subsequently on week 2, 3 and 4 in a duration-dependent manner ($p < 0.001$, one way ANOVA, $F = 58.14$; $p < 0.05$, Tukey's test).

3.8. Effects of OVX on gnrh2 expression

In the resting phase, OVX produced an overall significant effect on the expression of *gnrh2* mRNA (Fig. 6; $p < 0.001$, one way ANOVA; $F = 12.67$). The expression increased in the first week of OVX in comparison to initial and sham control groups but decreased subsequently on week 2, 3 and 4 duration-dependently ($p < 0.05$, Newman-Keuls' test). In the prespawning phase, there was a duration-dependent decrease in the transcript levels after OVX ($p < 0.001$, one way ANOVA; $F = 18.34$; $p < 0.05$, Newman-Keuls' test). In the sham groups, there was no significant effect in either season.

3.9. Effects of E_2 on gnrh2 expression in OVX and sham control fish

The administration of E_2 in 3-week ovariectomized fish increased the *gnrh2* transcript levels significantly compared to the ovariectomized-vehicle group (Fig. 7; $p < 0.001$, one way ANOVA; resting phase, $F = 76.32$, prespawning phase, $F = 65.10$; $p < 0.05$, Newman-Keuls' test). The magnitude of the increase was higher in the resting phase compared to the prespawning phase. On the other hand, the administration of E_2 in the sham control group decreased the transcript levels compared to the sham, sham-vehicle, OVX-vehicle and OVX-E_2 groups. The inhibition was higher in the prespawning phase.

3.10. Effects of testosterone on gnrh2 expression in OVX and sham control fish

In the resting phase, the administration of testosterone in 3-week OVX fish stimulated the *gnrh2* expression significantly over that of OVX and sham groups ($p < 0.001$, one way ANOVA, $F = 18.09$; $p < 0.05$,

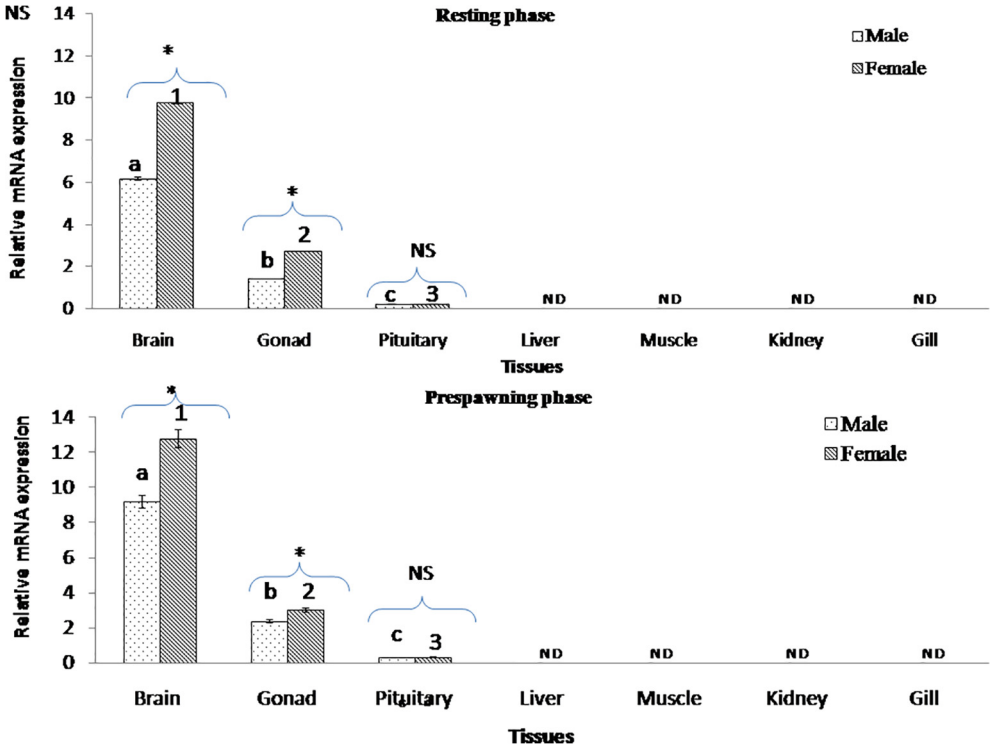

Fig. 3. Tissue expression of *gnrh2* mRNA (mean ± SEM, N = 5) in male and female *Heteropneustes fossilis* in the resting and prespawning phases. Note that the expression was found only in the brain, gonads and pituitary. The expression levels are relative values compared to the expression level of *β-actin* mRNA. Groups with different letters/numbers are significantly different ($p < 0.001$, one way ANOVA; $p < 0.05$, Newman-Keuls' test). The superscripted letters or numbers indicate comparisons among the male (a, b) and female (1, 2) tissues. Asterisk indicates significant difference between male and female tissues. ND – not detected. NS - not significant.

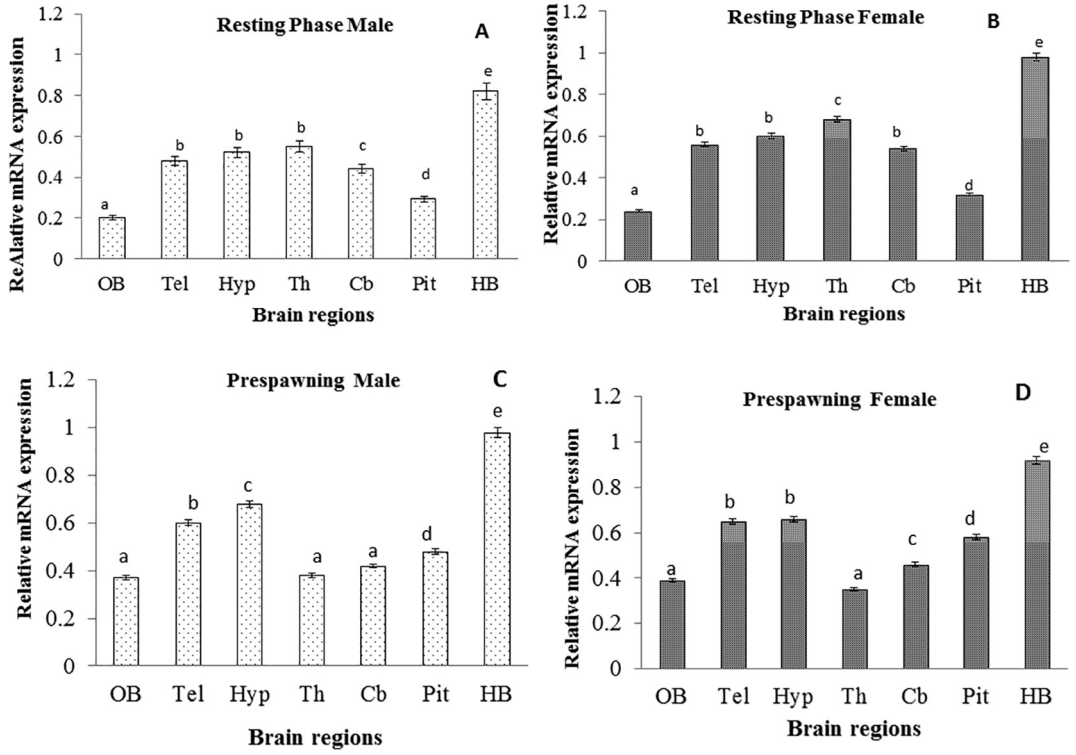

Fig. 4. Expression of *gnrh2* mRNA in different regions of the brain of male and female *Heteropneustes fossilis* (mean ± SEM; n = 5) in the resting and prespawning phases. The expression levels are relative values compared to the expression level of *β-actin* mRNA. Groups that bear the same letters are not significantly different ($p < 0.001$, one way ANOVA; $p < 0.05$, Newman-Keuls' test). OB-olfactory bulb, Tel– telencephalon, Hyp – hypothalamus, Th - thalamus, Cb- cerebellum, Pit-pituitary, HB- hindbrain- medulla oblongata.

Newman-Keuls' test). In the prespawning phase, testosterone increased the *gnrh2* expression over that of OVX and almost restored the level to that of the sham groups (Fig. 8; $p < 0.001$, one way ANOVA, $F = 16.12$; $p < 0.05$, Newman-Keuls' test). In the sham control group, testosterone decreased significantly the transcript levels ($p < 0.05$) and the inhibition was higher in the prespawning phase.

3.11. Effects of P_4 on gnrh2 expression in OVX and sham control fish

In the resting phase, the administration of P_4 in 3-week OVX fish

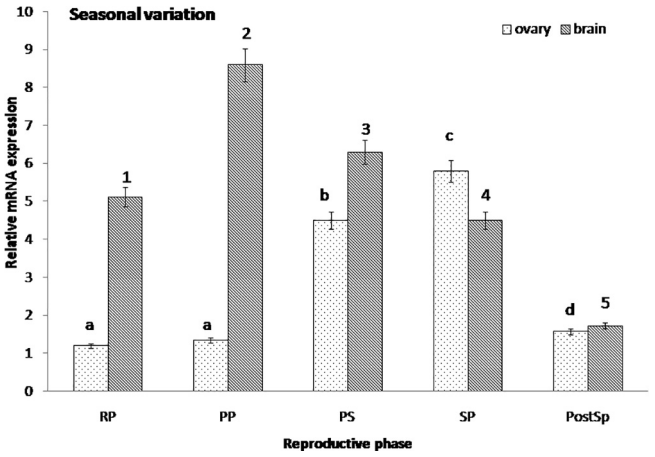

Fig. 5. Expression of *gnrh2* mRNA in the brain and ovary of the catfish *Heteropneustes fossilis* (mean ± SEM; N = 5) during different phases of the annual reproductive cycle. The expression levels are relative values compared to the expression level of β-actin mRNA. Groups that bear the same superscripted letters (ovary) or numbers (brain) are not significantly different ($p < 0.001$, one way ANOVA; $p < 0.05$, Newman-Keuls' test). RP – resting phase, PP – preparatory phase, PS – prespawning phase, SP – spawning phase, PostSP – post spawning phase.

increased the *gnrh2* expression significantly over that of OVX and sham groups ($p < 0.001$, one way ANOVA, $F = 19.07$; $p < 0.05$, Newman-Keuls' test). In the prespawning phase, testosterone increased the *gnrh2* expression over that of OVX and restored the transcript levels to that of the sham groups (Fig. 9; $p < 0.001$, one way ANOVA, $F = 15.08$; $p < 0.05$, Newman-Keuls' test). In the sham control group, P_4 decreased significantly the transcript levels ($p < 0.05$) with a higher inhibition in the prespawning phase.

4. Discussion

4.1. Gnrh2 nucleotide and protein sequences

The cloned *gnrh2* full length cDNA is 611 bp long containing an ORF of 261 bp and a polyadenylation signal AATAAAA at the 3′ UTR. The ORF encodes an 86 aa long prepro-Gnrh2 protein. Gnrh2 is conserved in all vertebrates from fish to mammals (Fernald and White, 1999), and was first isolated in chicken (chicken Gnrh2). The sequence comparison of the deduced prepro-Gnrh2 of *H. fossilis* has conserved features of the Gnrh2 precursor of other teleosts and other vertebrates. In the phylogenetic tree, the *H. fossilis* Gnrh2 precursor aligned with the African catfish Gnrh2 precursor (94% sequence identity) in the Ostariophysan subclade, distinct from the Acanthopterygian subclade. The African catfish *gnrh2* cDNA is shorter (546 bp) with a short ORF (258 bp) sequence (Bogerd et al., 1994) but encodes a protein of 86 aa, like the *H. fossilis* protein. On the other hand, the African catfish Gnrh1 is an 80 aa-long protein encoded by a cDNA of 351 bp and ORF of 240 bp. Two types of Gnrh1 are recognized in the African catfish based on nucleotide/amino acid differences in the GAP domain. Our efforts to clone or retrieve the *gnrh1* sequence have not succeeded yet and the cloning work is in progress. Interestingly, the cyprinid fathead minnow (*Pimephales promelas*) and the protogynous black sea bass (*Centropristis striata*) lack a *gnrh1* gene and have only the *gnrh2* and *gnrh3* subtypes (Filby et al., 2008; Morin et al., 2015), indicating that the Gnrh system is highly diverse in teleosts.

4.2. Tissue and sex dimorphic expression

Teleosts show wide variations in the tissue expression of Gnrh

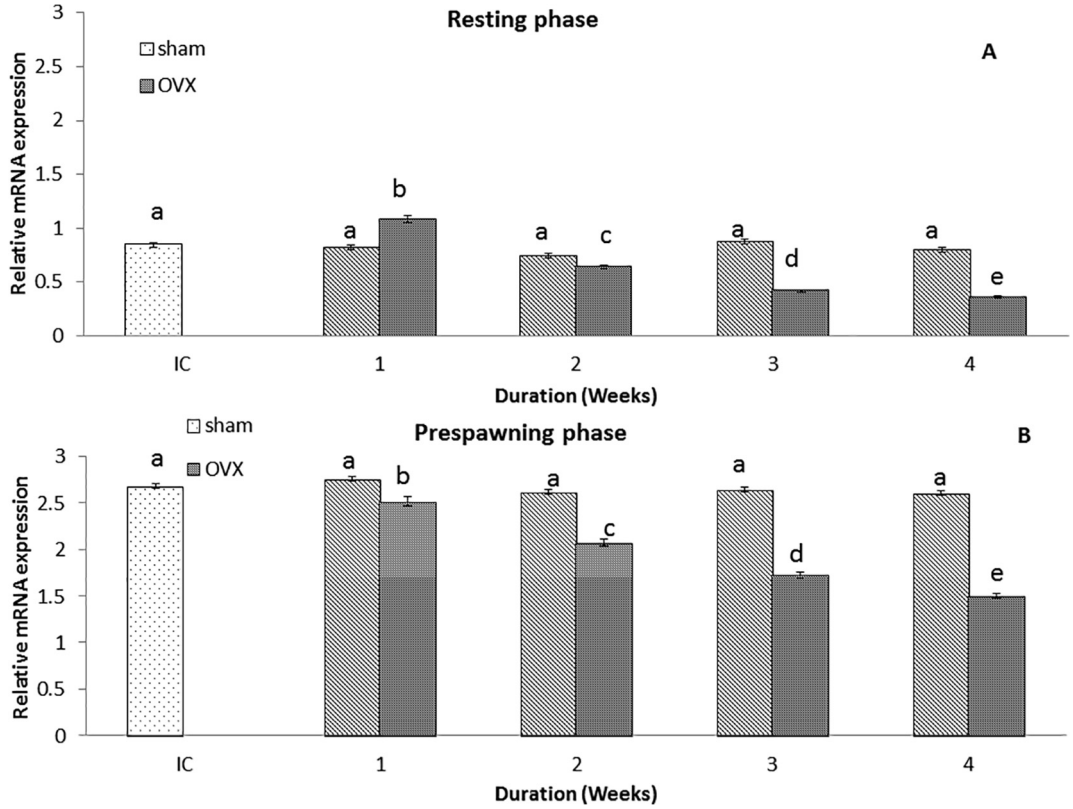

Fig. 6. Effects of ovariectomy on *gnrh2* mRNA expression in *Heteropneustes fossilis* (mean ± SEM, N = 5) in the resting and prespawning phases. The data were analyzed by one way ANOVA ($p < 0.001$) and Newman-Keuls' test ($p < 0.05$). Groups with different superscripted letters are significant from the sham control or ovariectomized group.

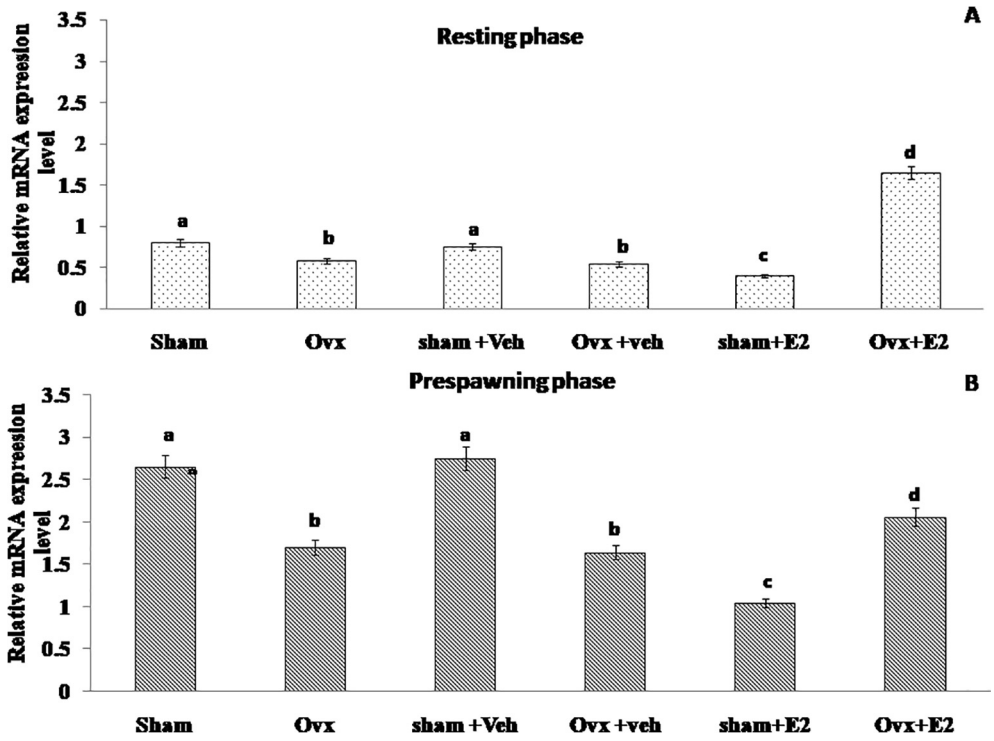

Fig. 7. Effects of E_2 on *gnrh2* mRNA expression in 3-week ovariectomized catfish *Heteropneustes fossilis* in the resting and prespawning phases (mean ± SEM, N = 5). Other details are as in Fig. 6 legend.

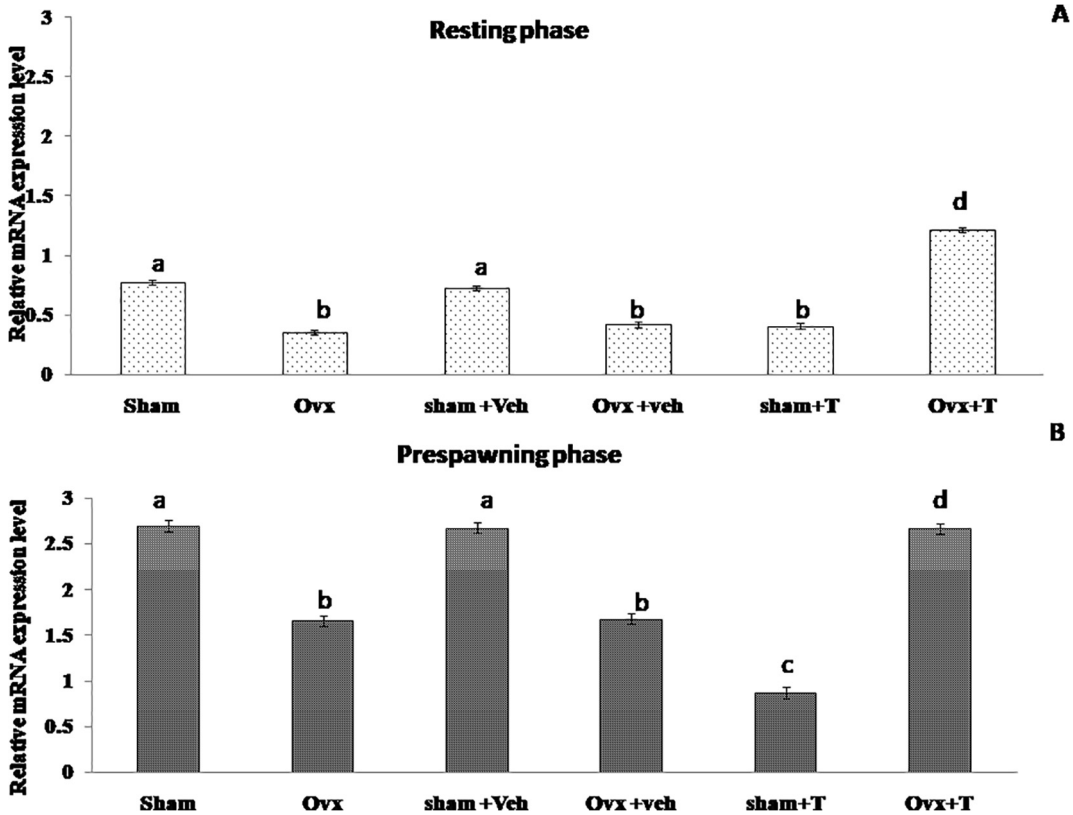

Fig. 8. Effects of testosterone on *gnrh2* mRNA expression in 3-week ovariectomized catfish *Heteropneustes fossilis* in the resting and prespawning phases (mean ± SEM, N = 5). Other details are as in Fig. 6 legend.

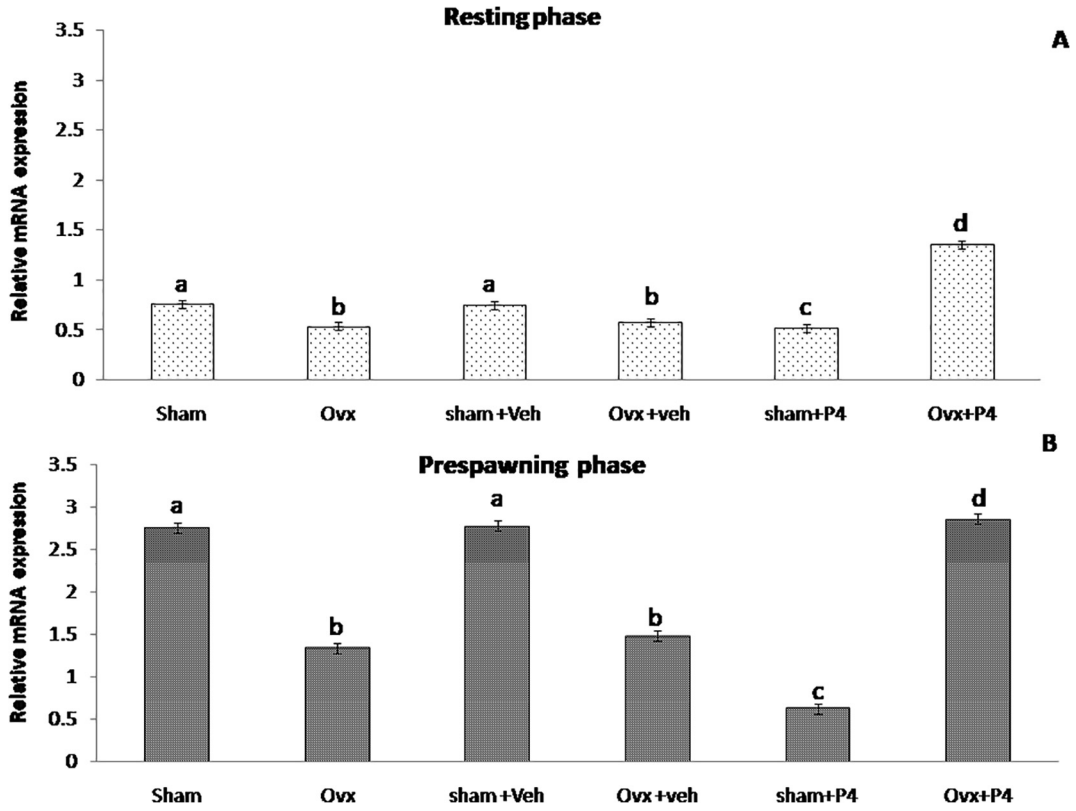

Fig. 9. Effects of progesterone (P_4) on *gnrh2* mRNA expression in 3-week ovariectomized catfish *Heteropneustes fossilis* in the resting and prespawning phases (mean ± SEM, N = 5). Other details are as in Fig. 6 legend.

transcripts or proteins (Chang and Pemberton, 2018). In *H. fossilis*, the *gnrh2* transcripts were expressed highly in the brain, moderately in the gonads and low in the pituitary. Other peripheral tissues did not show any expression. The expression is higher in the brain and sex dimorphic with a female bias. In goldfish (*Carassius auratus*), both salmon *gnrh* and *gnrh2* were expressed in the brain and ovary (Lin and Peter, 1996). Later two forms of *gnrh2* mRNA (mRNA-1 and mRNA-II) were characterized in this species and *gnrh2* mRNA-I, but not *gnrh2* mRNA-II, was expressed in the ovary and testis (Yu et al., 1998). In *Haplochromis burtoni*, 3 forms of Gnrh were characterized, which have differential peripheral tissue expression (White and Fernald, 1998). In *Mugil cephalus* (Nocillado et al., 2007), *gnrh1* expression was reported in the brain during the early perinucleolar stage and in the ovary during advanced postvitellogenic stage. Conversely, the authors reported a lack of expression of *gnrh2* or *gnrh3* in the ovary and uncertain expression of *gnrh1* and *gnrh2* in the pituitary. In the fathead minnow (*Pimephales promelas*), *gnrh2* is expressed only in the brain, testis and ovary along with the *gnrh3* type (Filby et al., 2008). In the protogynous black sea bass *Centropristis striata*, brain has consistently shown the expression of *gnrh2* and *gnrh3* in small, medium and large sized fish while the gonads showed the expression of *gnrh2* in small and medium sized females and large sized males (Morin et al., 2015). Zhao et al. (2017) reported that out of the 3 forms of *gnrh* genes described in the turbot (*Schophthalmus maximus*), *gnrh2* expression was restricted to the brain along with the other two forms while *gnrh1* (*sbgnrh*) was expressed in higher amounts in the pituitary, gonads, skin and stomach. In the catfish, the *gnrh2* expression was consistent in the brain, ovary and testis and dimorphic.

4.3. Brain regional expression

Gnrh2 neurons are non placodal in origin and localized in the midbrain in contrast to Gnrh1 and/or Gnrh3 neurons, which have a placodal (olfactory) origin and distributed in the forebrain (Chen and Fernald, 2008; Chang and Pemberton, 2018). In agreement with the exclusive or the highest midbrain distribution of Gnrh2 transcripts or peptide in the teleost brain (Okuzawa et al., 1990; Schulz et al., 1993; Bogerd et al., 1994; Powell et al., 1994; Goos et al., 1997; White and Fernald, 1998; Senthilkumaran et al., 1999; Gonzalez-Martinez, et al., 2002; Xia et al., 2014; Zhao et al., 2017), the midbrain-hindbrain showed the highest transcript expression in the catfish. Also, the *gnrh2* transcripts were detected in the forebrain regions to varying abundance. In fact, the expression of *gnrh2* in regions other than the midbrain tegmentum shows great species variations. Brain regional expression also varied with the sexes especially in the hind brain. Two *gnrh2* mRNAs were characterized in goldfish and by RT-PCR assay and Southern blot analysis, hybridization signals of the two forms were detected in all brain areas (olfactory bulb and tract, telencephalon, hypothalamus, optic tectum–thalamus, and posterior brain), but with a difference in the signal intensity (Lin and Peter, 1996; Yu et al., 1998). Intra cerebro-ventricular injection of corticotrophin-releasing hormone (CRH) up regulated the *gnrh2* expression in the goldfish hypothalamus (Kang et al., 2011). In the fathead minnow, Filby et al. (2008) reported that the expression of *gnrh2* was predominantly in the region that included the hypothalamus and midbrain tegmentum (approximately 250-fold higher expression than in the forebrain regions, optic nerves, optic tectum, and hindbrain regions, where only very low levels of expression were observed). In the stickleback *Gasterosteus aculeatus*, *gnrh2* mRNA levels were detected in the cerebellum, thalamus and hypothalamus. However, the expression in the hypothalamus was far less than that in the thalamus (Shao et al., 2015). In the catfish, the *gnrh2* expression increased in the telencephalon and hypothalamus in the gonad-active prespawning phase compared to that of the resting phase. On the other hand, *in situ* hybridization or immunocytochemical study did not indicate a forebrain distribution of Gnrh2 in the African catfish (Schulz et al., 1993; Bogerd et al., 1994). The wide spread expression of *gnrh2* is intriguing since Gnrh2 neurons are localized in the

midbrain tegmentum and the neuronal projections (fiber terminals) to other parts of the brain especially the midbrain, hindbrain, and third ventricle regions (see Chen and Fernald, 2008 for references) may not contain transcripts. Localization studies are required to confirm the forebrain expression in the catfish.

The expression of Gnrh2 transcripts/peptide show great variations in the pituitary of teleosts ranging from almost undetectable levels to high contents (Chang and Pemberton, 2018). The pituitary projection of the Gnrh2 fibers has been traced to the midbrain Gnrh2 neurons in the goldfish and zebrafish (Kim et al., 1995; Xia et al., 2014). In the gilthead seabream *Sparus aurata*, the pituitary abundance varied in a ratio of 100:10:1 for Gnrh1: Gnrh2: Gnrh3, respectively (Gothilf et al., 1995). In Senegalese sole (*Solea senegalensis*), the pituitary content of Gnrh2 was greater than those of Gnrh1 and Gnrh3 (Guzman et al., 2009). In the African catfish, Gnrh1 is 37-folds greater in the pituitary than Gnrh2 (Schulz et al., 1993). There was no expression in the pituitary of fathead minnow (Filby et al., 2008). In *H. fossilis*, the expression level was low compared to the brain or gonads, and dimorphic and higher in the prespawning phase.

4.4. Seasonal expression in brain and ovary

In *H. fossilis*, *gnrh2* transcript levels showed significant seasonal variations in the brain and ovary, and followed opposite patterns. The transcript levels were significantly higher in the brain in all phases but attenuated in the post spawning phase. In the brain, the *gnrh2* transcripts peaked in the preparatory phase and decreased during the progress of ovarian recrudescence to the lowest level in the postspawning phase. This may be due to decreased transcript production or increased translational activity. The catfish pattern is different from what was described in other species. In turbot, the expression of brain *gnrh2* was low at stage III (primary spematocytes) and high from stage IV to VI (secondary spermatocytes - spermatids + spermatozoa - spermatozoa) in males and low at stage III (early vitellogenesis) and high from stage IV to VI (late vitellogenesis-ovulated oocytes- postovulatory follicles) in females (Zhao et al., 2017). In the red seabream *Pagrus major*, Gnrh2 levels in the brain were higher in the regressed phase and remained lower during the spawning phase (Senthilkumaran et al., 1999), while the *gnrh2* mRNA levels showed a slight increase in the spawning phase (Okuzawa et al., 2003). In the stickleback, *gnrh2* mRNA levels in the thalamus were significantly higher in mature males than those in post-breeding males (Shao et al., 2015). The brain expression pattern in the catfish may suggest an early reproductive role for Gnrh2 in stimulating gonadotropin or somatotropin release. In the African catfish, Gnrh2 fibers were localized along with Gnrh1 fibers and showed a higher LH-releasing capacity compared to that of catfish Gnrh1 in *in vitro* pituitary perifusion systems (Schulz et al., 1993). The LH stimulating action of Gnrh2 has been also reported in other teleosts (see Chang and Pemberton, 2018 for references).

In the ovary, the expression of *gnrh2* increased progressively from the resting phase to peak in the spawning phase and decreased in the postspawning phase, suggesting a functional role of the peptide in ovarian activity. The sharp decline in the expression after spawning may indicate a role related to final oocyte maturation and ovulation. In rainbow trout, salmon Gnrh2 (sGnrh2) is the major Gnrh form in testis and ovary, which showed a reproductive stage-specific expression (Uzbekova et al., 2001). The authors reported that mRNA was highly expressed before the start of spermatogenesis, disappeared at stage II and then increased progressively up to stage VI of spermatogenesis. In the ovary, the gene expression was high in immature pre-vitellogenic fish and progressively decreased throughout vitellogenesis. At ovulation, it reached the peak and declined after stripping of the eggs. The authors related these changes to anti-proliferative and meiosis resumption action of sGnRH during gametogenesis. The *gnrh2* expression in the ovary of the catfish suggests a paracrine/autocrine role of the peptide in ovarian steroidogenesis and oocyte growth and ovulation. These aspects have to be investigated in future experimental studies.

4.5. Steroid modulation of gnrh2 expression

It is well established that gonadal steroid hormones exert feedback control on Gnrh secretion in vertebrates (Tilbrook and Clarke, 2001; Lee et al., 2008; Brayman et al., 2012). Such studies are focused more on the hypophysiotropic Gnrh form (Gnrh1) that has major implications in most fish on gonadotropin release. Ovariectomy and steroid replacement are classical methods to evaluate the feedback actions of steroids and the approach was used in the catfish previously (Senthilkumaran and Joy, 1994). In the resting phase, OVX resulted in biphasic effects: a significant stimulation of *gnrh2* expression on week 1 and a progressive and significant inhibition of the gene expression on week 2, 3 and 4. In the prespawning phase, the *gnrh2* expression decreased significantly and progressively at all duration. The OVX-induced reduction was higher in the resting phase: on week 4 the inhibition was about 50% compared to that in the prespawning phase. Since OVX resulted in significant decreases in plasma E_2, testosterone and P_4 levels, the changes in the mRNA expression could be related to the altered steroid feedback relations. The data of the steroid replacement in 3-week OVX catfish show that steroids can elicit stimulatory effects on the gene expression. The E_2 replacement countered the OVX-induced reduction in both resting and prespawning phases but the magnitude of the response varied: in the resting phase, E_2 produced more than a twofold increase in the transcript level elevating the level sharply over the sham control groups. In the prespawning phase though E_2 countered the OVX effect, the increase was significantly lower than that of the sham control groups. It may be explained that in the gonad resting phase, the E_2 level is low and the exogenously administered steroid increased its plasma titer and activated a positive feedback action resulting in the sharp rise in the gene expression. On the other hand, in the gonad active prespawning phase, the normal plasma E_2 titer is high so that the exogenously supplied steroid increased further the titer, which might have exerted a negative feedback effect on the transcript level. This argument is also supported by the sham control group data. In the sham group, E_2 decreased the transcript levels in both phases and the inhibition was greater in the prespawning phase. Thus E_2 may maintain both positive and negative feedback control on *gnrh2* expression depending on the reproductive stage of the catfish. There are very few studies on the estrogen control of *gnrh* gene expression in teleosts. Parhar et al. (2000) reported that the 3 forms of Gnrh (sGnrh, seabream Gnrh and chicken Gnrh2) are differentially controlled by sex steroids and thyroid hormone in immature male tilapia (*Oreochromis niloticus*). Estradiol benzoate, 11-ketotestosterone and triiodothyronine (T3) treatments had no influence on the midbrain *gnrh2* mRNA content or neuronal numbers, indicating that the regulatory mechanisms controlling Gnrh2 secretion may be different. In early vitellogenic female European sea bass (*Dicentrarchus labrax*), ovariectomy stimulated the hypothalamic *gnrh1* expression but not the pituitary *gnrh2-1a* expression, and E_2 replacement lowered the ovariectomy-induced stimulation of *gnrh1* expression to the control level (Alvarado et al., 2016).

Since OVX elicited a decrease in plasma P_4 and testosterone levels along with the female hormone E_2, we thought to investigate whether these intermediate steroids of estrogen synthesis pathway had any effect on *gnrh2* expression. In the 3-week OVX catfish, the administration of testosterone elicited similar effects except that in the prespawning phase, transcript abundance reached almost to the level of sham control groups. In the sham groups, testosterone decreased the transcript level, like E_2. Castration did not produce any significant effect on brain *gnrh2* expression in breeding male sticklebacks (Shao et al., 2015). In the European sea bass, no changes in mRNA levels of hypothalamic *gnrh1* or pituitary *gnrhr2-1a* genes were observed in males, castrated or testosterone implanted, in early testicular recrudescence (Alvarado et al., 2016). The P_4 treatment in the 3-week OVX and sham control fish

produced effects similar to that of testosterone and E_2. It could be argued that in the sham control group P_4 and testosterone effects may be indirect due to the conversions to E_2. Future studies using specific receptor blockers of E_2, P_4 and testosterone may contribute convincing evidence on the mode of action of steroids.

The question whether steroids act directly or indirectly to modify Gnrh secretion is debated; evidence for both direct and indirect actions are forthcoming in humans and animals (Fernald and White, 1999; Lee et al., 2008). In the teleost *Haplochromis burtoni*, putative binding sites for glucocorticoid, androgen, and P_4 receptors were identified in the 500 bp upstream of exon 1 of the 3 forms of *gnrh* genes but ER binding sites were not found (White and Fernald, 1998). In the rainbow trout *Onchorhynchus mykiss*, the forebrain Gnrh neurons did not show any immunoreactivity for ERs (Navas et al., 1995). On the other hand, the Gnrh-secreting hypothalamic cell line, GT1–7 neurons expressed both ER α and ERβ subtypes and E_2 (1 nM) down-regulated Gnrh mRNA levels to approximately 55% of basal levels over a 48 h time course. This effect was blocked by a potent ER antagonist (ICI 182,780) (Roy et al., 1999). In the indirect mechanism, kisspeptin neurons that abut on the Gnrh neurons are the major sites of estrogen negative and positive feedbacks (Marques et al., 2015).

4.6. Functional implications gnrh2 expression in brain and ovary

The expression of *gnrh2* in the brain and gonads implies that Gnrh2 is involved in brain and gonadal functions. In the catfish, the expression of *gnrh2* in the forebrain regions may be related to the control of neuroendocrine functions and feeding behaviour. In goldfish, Gnrh2 mediates the anorexigenic actions of CRH and α-melanocyte-stimulating hormone and inhibits food intake (Kang et al., 2011). In the European sea bass *D. labrax*, Gnrh2 stimulates nocturnal melatonin secretion in the pineal organ (Servili et al., 2010). The expression of *gnrh2* in the pituitary may suggest a hypophysiotropic role. In the gonads, the expression of *gnrh2* and its seasonal variation are indicative of a role in gametogenesis and spawning.

5. Conclusions

The present study reported cloning and characterization of a *gnrh2* precursor cDNA in the Ostariophysan catfish and demonstrated the highly conserved nature of the deduced protein. The *gnrh2* mRNA expressed only in the brain, gonads and pituitary. In the brain, the *gnrh2* transcripts are expressed extensively suggesting varied region-specific functions. In the gonads, the expression of *gnrh2* transcripts may indicate a paracrine/autocrine role in gonadal function. Brain *gnrh2* expression is modulated by feedback actions of gonadal steroids. The pituitary expression of *gnrh2* suggests a hypophysiotropic role for the peptide. The present data will form a basis for future studies aiming at localization of the gene/peptide, and defining the peptide functions and regulatory mechanisms.

Acknowledgements

The research work was supported by a grant from DST-SERB, New Delhi (EMR/2016/002751 dated 27-03-2017). We are grateful to Coordinator, DBT-BHU ISLS for the use of ABI Prism 7500 thermal cycler (Applied Biosystems, Foster, CA USA) for the qPCR assay. BS is the recipient of a DBT-TATA innovation fellowship. KPJ is INSA Emeritus Scientist.

Conflict of interests

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.021.

References

Alvarado, M.V., Servili, A., Molés, G., Gueguen, M.M., Carrillo, M., Kah, O., Felip, A., 2016. Actions of sex steroids on kisspeptin expression and other reproduction-related genes in the brain of the teleost fish European sea bass. J. Exp. Biol. 219, 3353–3365. https://doi.org/10.1242/jeb.137364.

Bogerd, J., Li, K.W., Janssen-Dommerholt, C., Goos, H.J.Th., 1992. Two gonadotropin-releasing hormones from African catfish *(Clarias gariepinus)*. Biochem. Biophys. Res. Commun. 187, 127–134.

Bogerd, J., Zandbergen, T., Andersson, E., Goos, H., 1994. Isolation, characterization and expression of cDNAs encoding the catfish-type and chicken-11-type gonadotropin-releasing-hormone precursors in the African catfish. Eur. J. Biochem. 222, 541–549.

Brayman, M.J., Pepa, P.A., Berdy, S.E., Mellon, P.L., 2012. Androgen receptor repression of GnRH gene transcription. Mol. Endocrinol. 26, 2–13.

Chang, J.P., Pemberton, J.G., 2018. Comparative aspects of GnRH-Stimulated signal transduction in the vertebrate pituitary - contributions from teleost model systems. Mol. Cell. Endocrinol. 463, 142e167.

Chaube, R., Mishra, S., 2012. Brain steroid contents in the catfish *Heteropneustes fossilis*: Sex and gonad stage-specific changes. Fish Physiol. Biochem. 38, 757–767. https://doi.org/10.1007/s10695-011-9558-0.

Chaube, R., Rawat, A., Inbaraj, R.M., Bobe, J., Guiguen, Y., Fostier, A., Joy, K.P., 2017. Identification and characterization of a catechol-o-methyltransferase cDNA in the catfish *Heteropneustes fossilis*: tissue, sex and seasonal variations, and effects of gonadotropin and 2-hydroxyestradiol-17β on mRNA expression. Gen. Comp. Endocrinol. 246, 129–141.

Chen, C.-C., Fernald, R.D., 2008. GnRH and GnRH receptors: distribution, function and evolution. J. Fish Biol. 73, 1099–1120. https://doi.org/10.1111/j.1095-8649.2008.01936.x.

Cho, B.N., Seong, J.Y., Cho, H., Kim, K., 1994. Progesterone stimulates GnRH gene expression in the hypothalamus of ovariectomized, estrogen treated adult rats. Brain Res. 652, 177–180.

Fernald, R.D., White, R.B., 1999. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front. Neuroendocrinol. 20, 224–240.

Filby, A.L., van Aerle, R., Duitman, J.W., Tyler, C.R., 2008. The kisspeptin/gonadotropin-releasing hormone pathway and molecular signaling of puberty in fish. Biol. Reprod. 78, 278–289. https://doi.org/10.1095/biolreprod.107.063420.

Frohman, M.A., Dush, M.K., Martin, G.R., 1988. Rapid production of full-length cDNAs from rare transcripts amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. U.S.A. 85, 8998–9002.

Gonzalez-Martınez, D., Madigou, T., Zmora, N., Anglade, I., Zanuy, S., Zohar, Y., Elizur, A., Muñoz-Cueto, J.A., Kah, O., 2001. Differential expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). J. Comp. Neurol. 429, 144–155.

Gonzalez-Martinez, D., Zmora, N., Mañanos, E., Saligaut, D., Zanuy, S., Zohar, Y., Elizur, A., Kah, O., Muñoz-cueto, J.A., 2002. Immunohistochemical localization of three different prepro-GnRHs in the brain and pituitary of the European sea bass (*Dicentrarchus labrax*) using antibodies to the corresponding GnRH-associated peptides. J. Comp. Neurol. 446, 95–113.

Goos, H.J.Th., Bosma, P.T., Bogerd, J., Tensen, C.P., Li, K.W., Zandbergen, M.A., Schulz, R.W., 1997. Gonadotropin-releasing hormones in the African catfish: molecular forms, localization, potency and receptors. Fish Physiol. Biochem. 17, 45–51.

Gothilf, Y., Elizur, A., Chow, M., Chen, T.T., Zohar, Y., 1995. Molecular cloning and characterization of a novel gonadotropin-releasing hormone from the gilthead seabream (*Sparus aurata*). Mol. Mar. Biol. Biotechnol. 4, 27e35.

Guzman, J.M., Rubio, M., Ortiz-Delago, J.B., Klenke, U., Kight, K., Cross, I., Sanchez-Ramos, I., Riaza, A., Robordinos, L., Saraquete, C., Zohar, Y., Mananos, E.K., 2009. Comparative gene expression of gonadotropins (FSH and LH) and peptide levels of gonadotropin-releasing hormones (GnRHs) in the pituitary of wild and cultured Senegalese sole (*Solea senegalensis*) broodstocks. Comp. Biochem Physiol. A Mol. Integr. Physiol. 153 266e267.

Herbison, A.E., 1998. Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrine Rev. 19, 302–330.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Kah, O., Lethimonier, C., Somoza, G., Guilgur, L.G., Vaillant, C., Lareyre, J.J., 2007. GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. Gen. Comp. Endocrinol. 153, 346–364.

Kang, K.S., Shimizu, K., Azuma, M., Ui, Y., Nakamura, K., Uchiyama, M., Matsuda, K., 2011. Gonadotropin-releasing hormone II (GnRH II) mediates the anorexigenic actions of α-melanocyte-stimulating hormone (α-MSH) and corticotropin-releasing hormone (CRH) in goldfish. Peptides 32, 31–35.

Karigo, T., Oka, Y., 2013. Neurobiological study of fish brains give insights into the nature of gonadotropin-releasing hormone 1–3 neurons. Front. Endocrinol. (Lausanne) 4, 177. https://doi.org/10.3389/fendo.2013.00177.

Kim, M.H., Oka, Y., Amano, M., Kobayashi, M., Okuzawa, K., Hasegawa, Y., Kawashima, S., Suzuki, Y., Aida, K., 1995. Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. J. Comp. Neurol. 356, 72–82.

Lee, V.H.Y., Lee, L.T.O., Chow, B.K.C., 2008. Gonadotropin-releasing hormone: regulation of the GnRH gene. FEBS J. 275, 5458–5478.

Lethimonier, C., Madigou, T., Munoz-Cueto, J.A., Lareyre, J.J., Kah, O., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen. Comp. Endocrinol. 135, 1–16.
Lin, X.W., Peter, R.E., 1996. Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. Gen. Comp. Endocrinol. 101, 282–296.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDCT method. Methods 25, 402–408.
Marques, P., Skorupskaite, K., Rozario, K.S., Anderson, R.A., George, J.T., 2015. Physiology of GnRH and gonadotropin secretion. In: De Groot, L.J., Chrousos, G., Dungan, K. (Eds.), Endotext [Internet]. South Dartmouth (MA). MDText.com, Inc., The National Library of Medicine, National Institutes of Health.
Mohamed, J.S., Khan, I.A., 2006. Molecular cloning and differential expression of three GnRH mRNAs in discrete brain areas and lymphocytes in red drum. J. Endocrinol. 188, 407–416.
Mohamed, J.S., Thomas, P., Khan, I.A., 2005. Isolation, cloning, and expression of three prepro-GnRH mRNAs in Atlantic croaker brain and pituitary. J. Comp. Neurol. 488, 384–395.
Morin, S.J., Decatur, W.A., Breton, T.S., Marquis, T.J., Hayes, M.K., Berlinsky, D.L., Sower, S.A., 2015. Identification and expression of GnRH2 and GnRH3 in the black sea bass (*Centropristis striata*), a hermaphroditic teleost. Fish Physiol. Biochem. 41, 383–395. https://doi.org/10.1007/s10695-014-9990-z.
Navas, J.M., Anglade, I., Bailhache, T., Pakdel, F., Breton, B., Jego, P., Kah, O., 1995. Do gonadotrophin releasing hormone neurons express estrogen receptors in the rainbow trout? A double immunohistochemical study. J. Comp. Neurol. 363, 461–474.
Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E., Sherwood, N.M., 1992. Primary structure of two forms of gonadotropin releasing hormone, one distinct and one conserved, from catfish brain. Mol. Cell. Neurosci. 3, 17–22.
Nocillado, J.N., Levavi-Sivan, B., Carrick, F., Elizur, A., 2007. Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin- releasing hormones (GnRH), and dopamine receptor D2 (*drd2*) in pubertal female grey mullet, *Mugil cephalus*. Gen. Comp. Endocrinol. 150, 278–287.
Okubo, K., Nagahama, Y., 2008. Structural and functional evolution of gonadotropin releasing hormone in vertebrates. Acta Physiol. 193, 3–15.
Okuzawa, K., Amano, M., Kobayashi, M., Aida, K., Hanyu, I., Hasegawa, Y., Miyamoto, K., 1990. Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. Gen. Comp. Endocrinol. 80, 116–126.
Okuzawa, K., Gen, K., Bruysters, M., Bogerd, J., Gothilf, Y., Zohar, Y., Kagawa, H., 2003. Seasonal variation of the three native gonadotropin-releasing hormone messenger ribonucleic acids levels in the brain of female red seabream. Gen. Comp. Endocrinol. 130, 324–332.
Pak, T.R., Chung, W.C., Roberts, J.L., Handa, R.J., 2006. Ligand-independent effects of estrogen receptor beta on mouse gonadotropin-releasing hormone promoter activity. Endocrinology 147, 1924–1931.
Parhar, I.S., 1997. GnRH in tilapia: three genes, three origins and their roles. In: Parhar, I.S., Sakuma, Y. (Eds.), GnRH Neurons: Gene to Behavior. Brain Shuppan, Tokyo, pp. 99–122.
Parhar, I.S., Soga, T., Sakuma, S., 2000. Thyroid hormone and estrogen regulate brain region-specific messenger ribonucleic acids encoding three gonadotropin-releasing hormone genes in sexually immature male fish, *Oreochromis niloticus*. Endocrinology 141, 1618–1626.
Powell, J.F.F., Zohar, Y., Elizur, A., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Lovejoy, D.A., Sherwood, N.M., 1994. Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proc. Nat. Acad. Sci. U.S.A. 91, 12081–12085.
Radovick, S., Ticknor, C.M., Nakayama, Y., Notides, A.C., Rahman, A., Weintraub, B.D., Cutler Jr., G.B., Wondisford, F.E., 1991. Evidence for direct estrogen regulationof the human gonadotropin-releasing hormone gene. J. Clin. Invest. 88, 1649–1655.
Roch, G.J., Busby, E.R., Sherwood, N.M., 2014. GnRH receptors and peptides: skating backward. Gen. Comp. Endocrinol. 209, 118e134.
Rose, M.T., Henikoff, G.J., Henikoff, S., 2003. CODEHOP (COnsensus- DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucl. Acids Res. 13, 3763–3766.
Roy, D., Angelini, N.L., Belsham, D.D., 1999. Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ERalpha)- and ERbeta-expressing GT1-7 GnRH neurons. Endocrinology 140, 5045–5053.
Schulz, R.W., Bosma, P.T., Zandbergen, M.A., Van der Sanden, M.C., Van Dijk, W., Peute, J., Bogerd, J., Goos, H.J., 1993. Two gonadotropin-releasing hormones in the African catfish, *Clarias gariepinus*: localization, pituitary receptor binding, and gonadotropin release activity. Endocrinology 133, 1569–1577. https://doi.org/10.1210/en.133.4.1569.
Senthilkumaran, B., Joy, K.P., 1994. Effects of ovariectomy and estradiol replacement in hypothalamic serotonergic and monoamine oxidase activity in the catfish, *Heteropneustes fossilis*: a study correlating plasma oestradiol and gonadotropin levels. J. Endocrinol. 142, 193–203.
Senthilkumaran, B., Okuzawa, K., Gen, K., Ookura, T., Kagawa, H., 1999. Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. J. Neuroendocrinol. 11, 181–186.
Servili, A., Lethimonier, C., Lareyre, J.J., López-Olmeda, J.F., Sánchez-Vázquez, F.J., Kah, O., Muñoz-Cueto, J.A., 2010. The highly conserved gonadotropin-releasing hormone-2 form acts as a melatonin-releasing factor in the pineal of a teleost fish, the European sea bass *Dicentrarchus labrax*. Endocrinology 151, 2265–2275.
Shao, Y.T., Tseng, Y.C., Chang, C.H., Yan, H.Y., Hwang, P.P., Borg, B., 2015. GnRH mRNA levels in male three-spined sticklebacks, *Gasterosteus aculeatus*, under different reproductive conditions. Comp. Biochem. Physiol., Part A 180, 6–17.
Singh, V., Joy, K.P., 2009. Relative in vitro seasonal effects of vasotocin and isotocin on ovarian steroid hormone levels in the catfish *Heteropneustes fossilis*. Gen. Comp. Endocrinol. 162, 257–264.
Steven, C., Lehnen, N., Kight, K., Ijiri, S., Klenke, U., Harris, W.A., Zohar, Y., 2003. Molecular characterization of the GnRH system in zebrafish (*Danio rerio*): cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. Gen. Comp. Endocrinol. 133, 27–37.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.
Tilbrook, A.J., Clarke, I.J., 2001. Negative feedback regulation of the secretion and actions of gonadotropin- releasing hormone in males. Biol. Reprod. 64, 735–742.
Torgersen, J., Nourizadeh-Lillabadi, R., Husebye, H., Aleström, P., 2002. *In silico* and *in situ* characterization of the zebrafish (*Danio rerio*) *gnrh3* (sGnRH) gene. BMC Genomics 3, 25.
Uzbekova, S., Lareyre, J., Guiguen, Y., Ferrière, F., Bailhache, T., Breton, B., 2001. Expression of sGnRH mRNA in gonads during rainbow trout gametogenesis. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 129, 457–465. https://doi.org/10.1016/S1096-4959(01)00347-5.
Vickers, E.D., Laberge, F., Adams, B.A., Hara, T.J., Sherwood, N.M., 2004. Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, *Coregonus clupeaformis*. Biol. Reprod. 70, 1136–1146.
Volkoff, H., Peter, R.E., 1999. Actions of two forms of gonadotropin releasing hormone and a GnRH antagonist on spawning behavior of the goldfish *Carassius auratus*. Gen. Comp. Endocrinol. 116, 347–355.
White, R., Fernald, R., 1998. Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. Gen. Comp. Endocrinol. 112, 17–25.
Wolfe, A., Wu, S., 2012. Estrogen receptor-β in the gonadotropin-releasing hormone neuron. Semin. Reprod. Med. 30, 23–31.
Xia, W., Smith, O., Zmora, N., Xu, S., Zohar, Y., 2014. Comprehensive analysis of GnRH2 neuronal projections in zebrafish. Sci. Rep. 4, 3676. https://doi.org/10.1038/srep03676.
Yamamoto, N., Oka, Y., Kawashima, S., 1997. Lesions of gonadotropin-releasing hormoneimmunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. Neuroendocrinology 65, 403–412.
Yu, K.-L., He, M.-L., Chik, C.-C., Lin, X.-W., Chang, J.P., Peter, R.E., 1998. mRNA expression of gonadotropin releasing hormones (GnRHs) and GnRH receptor in goldfish. Gen. Comp. Endocrinol. 112, 303–311.
Zhao, C., Xu, S., Feng, C., Liu, Y., Yang, Y., Wang, Y., Xiao, Y., Song, Z., Liu, Q., Li, J., 2017. Characterization and differential expression of three GnRH forms during reproductive development in cultured turbot *Schophthalmus maximus*. Chin. J. Oceano. Limn. https://doi.org/10.1007/s00343-018-7068-y.