Seasonal expression and distribution of kisspeptin1 (kiss1) in the ovary and testis of freshwater catfish, *Clarias batrachus*: A putative role in steroidogenesis

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Highlights

- Kiss1 expresses in the theca and granulosa cells of ovarian oocyte and interstitial cells in testis of *Clarias batrachus*.
- Kisspeptin receptor antagonist (p234) treatment decreases production of 17β-estradiol in ovary and testosterone in testis.
- The p234 decreases the activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase.
- The p234 treatment halts the progression of oogenesis.
- The p234 arrests the testicular growth and spermatogenesis.

Abstract

The central role of kisspeptin (kiss) in mammalian reproduction is well established; however, its intra-gonadal role is poorly addressed. Moreover, studies investigating intra-gonadal role of kiss in fish reproduction are scanty, contradictory and inconclusive. The expression of *kiss1* mRNA has been detected in the fish brain, and functionally attributed to the regulation of reproduction, feeding and behavior. The *kiss1* mRNA has also been demonstrated in tissues other than the brain in some studies, but its cellular distribution and role at the tissue level have not been adequately addressed in fish. Therefore, an attempt was made in the present study to localize kiss1 in gonadal cells of the freshwater catfish, *Clarias batrachus.* This study reports the presence of kiss1 in the theca cells and granulosa cells of the ovarian oocytes and interstitial cells in the testis of the catfish. The role of kiss1 in the ovary and testis of the catfish was also investigated using kiss1 receptor (kiss1r) antagonist (p234). The p234 treatment decreased the production of 17β-estradiol in ovary and testosterone in the testis by lowering the activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase under both, *in vivo* as well as *in vitro* conditions. The p234 treatment also arrested the progression of oogenesis, as evident from the low number of advancing/advanced oocytes in the treated ovary in comparison to the control ovary. It also reduced the area and perimeter of the seminiferous tubules in the treated catfish testis. Thus, our findings suggest that kiss is involved in the regulation of gonadal steroidogenesis, independent of known endocrine/ autocrine/ paracine regulators, and thereby it accelerates gametogenic processes in the freshwater catfish.

Keywords: Kisspeptin; 3β-HSD; 17β-HSD; Sex steroids; Gametogenesis

1. Introduction

Vertebrate reproduction is classically controlled by interactive actions of hormones secreted from the hypothalamus, pituitary gland and gonad (HPG axis). The secreted hormones of this axis also regulate their own secretions through feedback mechanisms (Conn and Crowley, 1994; Ojeda et al., 2006). Gonadotropin-releasing hormone (GnRH), a decapeptide hypothalamic neurohormone, is a key regulator of reproductive activities in both the sexes of vertebrates (Ogawa et al., 2006). GnRH regulates the synthesis and release of gonadotropins; luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Kaiser et al., 1995). The LH and FSH once secreted induce gonads to produce sex-steroids (Backstrom et al., 1982; Burger et al., 2004; Marshall and Kelch, 1986; Wu et al., 1990), which ultimately trigger the commencement and progression of gonadal growth and maturation (Nagahama and Yamashita, 2008; Yaron et al., 2003).

In the recent past, kiss and its receptor have been reported as one of crucial regulators of hypothalamic GnRH secretion (Ogawa et al., 2012; Parhar et al., 2004; Selvaraj et al., 2012). Kiss, a neuropeptide, and its G-protein couple receptor (GPR54) are reported to influence activities of the hypothalamic–pituitary–gonadal axis (Greives et al., 2007) and act as a vital gatekeepers of reproduction (Seminara et al., 2003). Co-localization of kiss and GnRH in the hypothalamic nuclei suggests that kiss binds to its cognate receptor on GnRH, and thereby stimulates GnRH secretion (Messager et al., 2005; Tsutsui et al., 2010).

Lately, the signaling mechanism mediated by kiss1 and its receptor (kiss1r) is reported to regulate the attainment of puberty in mammals (Um et al., 2010). Inactivating mutations in the *kiss1r* gene causes hypogonadotropic-hypogonadism, a syndrome characterized by failure of pubertal development due to impaired secretion of FSH and LH (Karges and Roux, 2005; Topaloglu et al., 2012). Kiss1/Kiss1r are also known to mediate feedback effects of sex steroids on gonadotropin secretion (Clarke et al., 2015; Smith et al., 2013, 2006) and seasonal activities of reproduction in seasonally breeding animals (Greives et al., 2007; Revel et al., 2007; Smith and Clarke, 2007).

However, studies on kiss/GnRH in fishes are meager and poorly addressed, despite the fact that fish species display a diverse range and mode of reproduction and underlying regulatory mechanism, in contrast to mammalian species. Kiss and their receptor have been detected in some fish species, notably in the brain (Biran et al., 2008; Carrillo et al., 2009; Filby et al., 2008; Li et al., 2009; Mechaly et al., 2009; Mohamed et al., 2007; Nocillado et al., 2007; Parhar et al., 2004; van Aerle et al., 2008; Yang et al., 2010). Filby et al., 2008 have provided evidences supporting the role of neural kiss/kiss1r signaling in fish puberty. They have proposed a novel molecular mechanism of kiss/kiss1r action, and sex-steroid signaling in coordination and timing of the attainment of puberty in fish, which distinctly differs from the mechanism operative in mammals. The existence of kiss/kissr system is also reported in fish tissues other than brain such as testes, ovary, heart, muscle, stomach, intestine, spleen, liver, kidney, adipose tissue, pancreas, gills, eye and skin, to a variable extent depending on the species and kiss genes (Oakley et al., 2009; Saha et al., 2016). In teleost fishes, two kiss genes are reported, *kiss1* and *kiss2* derived from a common ancestral gene. Kiss is also reported to influence the commencement of puberty and reproduction in fish (Servili et al., 2011).

Although the expressions of *kiss1* mRNA and its receptor have been observed in gonadal tissue in some fishes, the existence of kiss peptide at a cellular level in the fish gonad is yet to

be elucidated. Likewise, the physiological significance of *kiss1* mRNA in gonadal tissue is poorly addressed. Therefore, the present study was undertaken to localize kiss1 expression and functions, if any, in the gonads of the Asian catfish, *Clarias batrachus*. Next, we observed the pattern of cellular and seasonal distribution of kiss1 protein in the ovary and testis. Further, we also aimed to establish its potential role in gonadal activities such as steroidogenesis and gametogenesis. This has relevance as *C. batrachus* is a seasonally breading freshwater Asian catfish, a widely consumed and economically important food fish in India.

2. Materials and methods

2.1. Reagents

Kiss1 antibody (PAS 3809) (against zebrafish kisspeptin1) and the control zebrafish kiss1 peptide (AS1560) were gifted to authors by Prof. Ishwar S. Parhar. Biotinylated goat anti-rabbit-IgG (secondary antibody) was procured from GeNei (cat No. 1110280011730) Bangalore, India. ABC Kit (elite kit PK-6100) was purchased from Vector Laboratories, Inc, Burlingame, CA, USA. 3, 3′-Diaminobenzidine tetrahydrochloride hydrate (DAB) and triton X-100 was obtained from Sigma-Aldrich, India. ELISA kits for 17β-Estradiol (cat no. DKO003) and testosterone (cat no. DKO002) were purchased from DiaMetra, Italy. Kiss1r antagonist (p234) was provided by Prof. R.P Millar, which was designed through extensive SAR studies on synthetic peptide custom synthesized by EZBio (Roseweir et al., 2009; Roseweir and Millar, 2013). Medium199 (Medium199 consists of Earle's salts, 25 mM HEPES buffer, L-Glutamine and Sodium bicarbonate, cat. no. AL094A) was obtained from Himedia Laboratories Pvt. Ltd., India. Other routine laboratory chemicals were acquired from Qualigens, Merck, SRL and HiMedia (AR Grade) through local authorized vendors.

2.2. Fish

Adult freshwater catfish, *C.batrachus* (weighing 85−90 g, and length 22−23 cm) were collected from a pond located in the suburb of Varanasi (25° 20′ N and 83° 00′ E), India, in the first week of each months starting from January to July covering gonadally inactive and active reproductive phases of the catfish, *i.e.*, January (early-quiescent), February (late-quiescent), March (early-recrudescence), April (mid-recrudescence), May-June (late-recrudescence), July (spawning phase). Nomenclature and phasing of reproductive cycle of the catfish is described elsewhere (Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018). Fish in each month were acclimated to laboratory conditions in cemented tanks (capacity of 200 L) under ambient photoperiod and temperature for two weeks and fed with minced goat liver *ad libitum*. All experiment were conducted in accordance with the guidelines of Institutional Animal Ethics and Care of Banaras Hindu University, India (approval letter No. F.Sc./IAEC/2016−17/1136) and as per the Guidelines of Committee for the Purpose of Control and Supervision of experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India, New Delhi, India, for Experimentation on Fishes.

After acclimation, fish were cold anaesthetized by immersing in ice-chilled water (6−7 °C) for 5−8 min following the method of Mittal and Whitear, 1978. The fishes were weighed and sacrificed to collect blood and gonadal tissues. Blood was collected in glass tubes through caudal puncture and centrifuged at 1370x g in refrigerated centrifuge to collect the serum. Their ovary and testis were also excised, rinsed in physiological saline and dried on Whatman

filter paper 1. One of the two ovaries and testes was fixed in aqueous Bouin's solution for 22 h for histological and immunohistochemical analyses, while other was stored at −80 °C till processed for the estimations of 17β-estradiol, testosterone and activity of steroidogenic enzymes.

2.3. Gonadal histology

Bouin's fluid-fixed testicular and ovarian tissues were processed for sectioning (6 μm thick) and haemotoxylin & eosin (H&E) staining following standard procedures described elsewhere (Singh et al., 2021; Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018). Briefly, the sections were mounted on 1% gelatin coated slides and dried on Leica hot plate (LEICA HI1220) at 40 °C overnight. Sections were then deparaffinized in xylene and hydrated using descending ethanol series. The sections were then stained with Ehrlich's hematoxylin solution for 45 min. The sections washed under running tap water for 30 min in coupling jar followed by differentiation with 1% acid water for 3−5 sec. The slides were again washed in running tap water for 60 min. The sections were then counterstained with eosin solution for 5 min. Thereafter, stained sections were dehydrated through ascending ethanol series, cleared in xylene and mounted with 1–2 drops of Lendrum's distrene oil butyl phthalate xylene (DPX), left overnight at room temperature before histological examination under Leica microscope (LEICA DM 2000).

2.4. Immunohistochemical localization of Kiss1

Localization of kiss1 in the ovary and testis was accomplished immunohistochemically. Briefly, the ovarian and testicular sections, after de-parafinization and hydration, were washed in phosphate buffer saline (PBS, 0.05 M, pH 7.5). The sections were then dropped in a cocktail of blocking solution containing 5% normal goat serum, 10 % TX-100 and 1% H_2O_2, at room temperature for 1 h. The sections were then incubated with polyclonal antibody kiss1 for 36 h at 4 °C. Control sections were incubated either with buffer omitting the primary antibody or kiss1 antibodies pre-adsorbed with kiss1 peptide (25 μg/mL buffer). These interventions resulted in complete loss of immunoreactivity. Positive controls were also processed similarly using zebrafish ovarian and testicular sections.

The sections were then washed with 0.1 % Triton X-100 in PBS for 1 h to remove unbound primary antibody and thereafter incubated with biotinylated secondary antibody (1:100) for 2 h at room temperature. Sections were washed and processed with Vectastain ABC elite kit containing 0.32 % Triton X-100 for 1 h and was washed subsequently in PBS for 1 h. To visualize primary antibody-binding sites, 3, 3′-Diaminobenzidine tetrahydrochloride hydrate (DAB- 0.025 % and 0.066 % hydrogen peroxide) was used as a chromogen which produced brown reaction product. The immunostained sections were not counterstained at any stage. Thereafter, sections were washed with PBS for 30 min and then processed for routine permanent mounting using DPX. Slides were examined under microscope with high resolution camera and images captured under bright field. The densitometric analyses of immunoreactive kiss1 of follicular cells in the ovarian oocytes and interstitial cells in the testis were analyzed by spot densitometry tool, Alpha EaseFC software (Alpha Innotech Corp., USA) in term of integrated density value (IDV) unit area-1 as described elsewhere (Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018; Singh and Lal, 2016, 2017; Kumar and Thakur, 2012; Kumar and Kumar Thakur, 2014). The IDV unit area-1 of follicular cells (theca and granulosa cells) of the ovarian follicles and testicular interstitial cells were calculated after subtracting the values of pre-adsorbed control from the

immunostained sections. Briefly, the images of immunoreactive kiss1 in gonadal sections were captured at 40x magnification using a microscope (Leica DM2000) attached with a CCD camera (Leica DFC295). The images were saved in JPEG (Joint Photographic Experts Group) format for further analysis of immunoreactive signals. The images of ovary and testis immunoreactive for kiss1 were opened in the alpha imager software and were converted into grayscale with the help of conversion tool provided with the software. Thereafter, spot denso analysis tool was selected which had an option of square object feature. The boxes of the size 1 × 1 cm and/or 0.5 × 0.5 cm for ovary and testis, respectively, were chosen using the square object feature of the software. Thereafter, the square box was randomly placed over kiss1 positive cells to obtain the final readings. The readings thus obtained were exported to excel sheet. The above method was used for a minimum of 4 slides (belonging to one ovary or one testis; each slide with 4 ovarian sections or 8 testicular sections). The four fields in each section were studied. Thus, the total of 48 readings of the single ovary and 96 of single testes were taken to calculate the average. Such process was repeated for five ovaries or testes in each group. Finally, the average of the five ovaries or testis was considered for further statistical analyses to show an increase or decrease in the immunoreactive signal of kiss1 in the ovary and testes in the present study. The method described above provides a semi-quantitative data on the immunoreactive signals, however, the limitations of the errors such as instrumentation not calibrated on immunostained sections without monochromatic light, may not be ruled out.

2.5. In vivo treatment of catfish with kiss1r antagonist (p234)

To evaluate the effect of p234 on steroidogenesis and gametogenesis, male and female *C.batrachus* were collected during the second week of gonadal mid-recrudescence phase (April). Following two-week acclimation, the catfish were sorted out in a narrow weight range (80−85 g), sexed into male and female, and divided into various groups (n = 8–10 fish in each). The control male and female catfish received vehicle fish saline (0.65 % fish saline); while different groups of each sex were injected with p234 antagonist at the dose levels 0.5 μg/100 g and 3 μg/100 g after immersing the fish in ice-chilled water (6−7 °C) for 5−8 min. Doses were selected based on the earlier report (Zmora et al., 2015). All injections were administered intramuscularly near the lateral line daily for 12 days. At the end of treatments, fish were weighed, testis and ovary was excised, one lobe of gonad was stored at −80 °C to determine steroids level, and activity of steroidogenic enzymes, while other lobe were fixed in Bouin's solution for histological studies.

2.6. In vitro treatments with kiss1r antagonist, p234

In order to decipher whether kiss1 influences steroid production, independent of known regulators of steroidogenesis in fishes, the effect of kiss1r antagonist was evaluated on steroidogenesis in ovarian and testicular explants. Experiments were also conducted during the mid-recrudescence phase of the reproductive cycle of the catfish. Fish were sacrificed, ovaries and testes were dissected out quickly (n = 5), cleaned and cut into small fragments (approximately 10 mg/fragment) in culture medium199. The culture medium was supplemented with 0.2 % $NaHCO_3$, penicillin 100IU/mL, streptomycin 100 μg/mL and 40 μg/mL gentamycin. After pre-incubation (three fragment/well) for about 3 h at 25 °C, the culture medium was discarded and gonadal explants were incubated with fresh medium (2 mL) containing 0.5, 5 and 50 nM of antagonist, p234, separately, under humidified atmosphere with 95 % air and 5% CO_2 at pH 7.4 for 24 h at 25 °C. Control gonadal tissue explants were incubated only in medium without antagonist concurrently. The treatments

were conducted in triplicate for each dose separately. All the experiments were repeated three times. At the end of the treatments, ovarian and testicular explants and their corresponding medium were collected separately and stored at −80 °C for further analyses of steroid in tissue fragments and culture medium.

2.7. Measurement of 17β-estradiol and testosterone

Analyses of 17β-estradiol and testosterone were performed using commercially available ELISA kit (DiaMetra, Italy) following the manufacturer's instructions. Briefly, 10 % gonadal tissue homogenate was prepared in phosphate buffer (0.01 M, pH 7.3). Then, 25 μL of homogenate and serum/culture medium were taken, separately, in the wells of ELISA plate. Thereafter, 200 μL of estradiol conjugate and 100 μL of testosterone conjugate was added in respective ovarian and testicular homogenate and serum/ culture medium, mixed and incubated at 37 °C for 2 h for estradiol and 1 h in case of testosterone. Following incubation, the content of each well was flicked properly and washed thrice with 300 μL of wash buffer provided with the kit. After washing, 100 μL of 3, 3′, 5, 5′- tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 30 min and 15 min in dark for estradiol and testosterone measurement, respectively. Reaction was then stopped by adding 100 μL of stop solution and the amount of enzyme complex was analyzed by measuring the optical density at 450 nm against a blank using the ELISA Reader (Multiskan, Thermo Labsystem). Blank wells were incubated only with TMB substrate. The sensitivity of the kit as per manufacturer's instruction is 0.008 ng/mL and 0.10 ng/mL, which is well within the detectable limits of estradiol and testosterone levels in fishes. The intra- and inter- assays variations reported in the kit is 5.4 % and 6.8 % for17β-estradiol and 6.4 % and 7.2 % for testosterone, respectively.

2.8. Determination of activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD)

Activity of steroidogenic enzymes like 3β-HSD and 17β-HSD were determined according to the original method of Wiebe, 1978 and Jarabak, 1969 respectively, with slight modification described elsewhere (Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018). Briefly, 10 % ovarian and testicular homogenate was prepared in buffered sucrose solution with potassium phosphate (5 mM) and EDTA (1 mM) and centrifuged at 12,000 *g* for 30 min at 4 °C. The supernatants collected were used to perform enzyme assay. For 3β-HSD activity, 250 mL of the supernatant was added in 3 mL cuvettes containing sodium phosphate buffer (0.1 M, pH7.5) and substrate dehydroepiandrosterone (0.3 mM). The samples were incubated for 5 min at 30 °C followed by addition of β-NAD at a concentration of 2.8 mM. Readings were taken every 15 s in a spectrophotometer at 340 nm against blank, without β-NAD.The enzyme activity was measured as change in absorbance with the time after addition of β-NAD. Enzyme activity was expressed as unit/hour/g ovary or testis. Similar procedure was adopted for the determination of 17β-HSD, except sodium phosphate buffer (0.44 mM, pH 10.2) and androstenedione (0.3 mM) was used as a substrate.

2.9. Morphometric analyses of ovarian and testicular sections

The counting of different types of oocytes in representative fields of ovarian sections were done manually while the morphometric analyses of testicular sections were done using Motic Image Plus V2.0 software, as per methods described elsewhere (Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018; Singh and Lal, 2016, 2017). Briefly, paraffin

embedded sections of the ovary and testis were processed for routine H/E staining. The sections were mounted on slides with DPX. Images of ovarian sections were captured at 10x and 40x by Leica DM2000. Following calibration of images, perinucleolar oocytes, oocytes-I, II and III were counted manually from representative fields (10^2mm/area) scattered in the ovarian sections. Three such areas from a single ovarian section and three sections of single ovary were taken into account. Similar counting was done for three catfish ovaries and the final counting was expressed in percent as Mean ± SEM. The area and perimeter of seminiferous tubules were also calculated in a similar fashion taking five catfish testis and expressed as Mean ± SEM.

2.10. Statistical analyses

Data pertaining to 17β-estradiol and testosterone concentrations, intensity of kiss1, area and perimeter of seminiferous tubules of testes are presented as Mean ± SEM (n = 5), while the counts of ovarian oocytes, activities of 3β-HSD & 17β-HSD as Mean ± SEM (n = 3).The levels of 17β-estradiol or testosterone are expressed as ng/g ovary or testis and ng/mL serum. For *in vitro* studies, the mean of five ovaries and testes or culture medium were calculated based on incubation in triplicates for every treatment conducted three times, independently. The enzyme activities are presented as unit/hour/g ovary or testis. All the data were analyzed through ANOVA followed by Duncan's multiple range test ($p < 0.05$) using SPSS software (SPSS v16.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Seasonal changes in ovarian morphology

Different types of oocyte of *C.batrachus* were categorized based on their specific features, described elsewhere (Sundararaj and Sehgal, 1970; Singh and Lal, 2016). The present catfish ovaries demonstrate asynchronous condition; different types of oocytes were present within the ovigerous folds. The perinucleolar oocytes have a large germinal vesicle with multiple nucleoli at its periphery and very less amount of cytoplasm. Non-yolky Oocytes-I are surrounded by somatic cells. Oocyte-I are larger than perinucleolar oocytes, and have a large germinal vesicle with substantial amount of cytoplasm. Oocytes-II (early-yolky oocytes) is larger in size with distinct cortical alveoli beneath the oocytes membrane. Oocytes II is surrounded by single layer of granulosa cells, and have very thin external thecal layer. Developing oocytes III are full of yolk granules and with well-developed granulosa and thecal layers. It was difficult to differentiate between granulosa and thecal cells in oocytes-I and II under the magnification used in this study. However, the follicular layers of the fully grown oocytes-III are highly developed where the granulosa and thecal cells are distinctly clear.

During the early- and late-quiescence phases, the ovaries showed predominantly perinucleolar oocytes and few oocytes-I (Fig. 1a', a" & b', b"). During the early-recrudescence phase, large number of oocytes-I was observed, and in some of the oocytes-I, the granulosa cells also started accumulating at the periphery of the oocytes membrane, suggesting the commencement of oogenesis (Fig. 1c'& c"). During the mid-recrudescence phase, in addition to oocytes-I, the oocytes-II started developing and the number of oocytes-II increased substantially. Low number of perinucleolar oocytes and oocytes-I were observed during this phase (Fig. 1d' & d"). In the late-recrudescence phase (May-June), the ovaries were populated by growing and fully grown oocytes-III with less number of perinucleolar

oocytes, oocytes-I and Oocytes-II (Fig. 1e', e", f' & f"). In the spawning period (July), dominance of oocytes-III was noticed. A good number of oocytes with germinal vesicles break down (GVBD), and spontaneously ovulated eggs were also observed during the spawning phase (Fig. 1g', g").

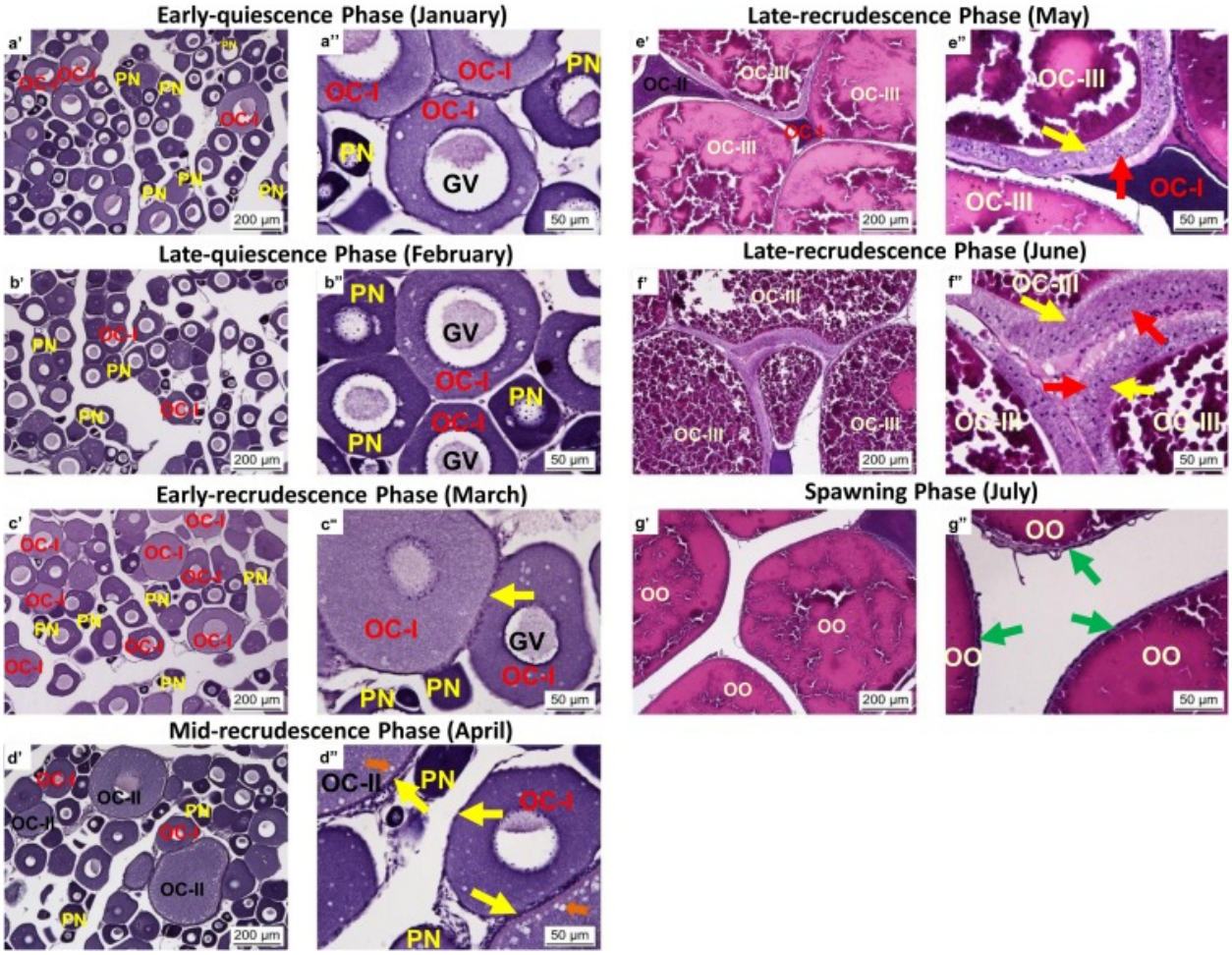

Fig. 1. Representative images of hematoxylin/eosin stained sections of the ovary of *Clarias batrachus* collected during the different reproductive phases, January to July (a' to g' and a" to g"). Early-quiescence phase (January) is represented as a', a", while b', b"; c', c"; d', d"; e', e"; f', f"; g', g", denotes late-quiescence (February), early-recrudescence (March), mid-recrudescence (April) for late-recrudescence (May), late-recrudescence (June), spawning (July), respectively. Note- Perinucleolar oocytes (PN), Oocytes-I (OC-I), Oocytes-II (OC-II), Oocytes-III (OC-III), Ovulated Oocytes (OO), Germinal vesicle (GV), Cortical Alveoli (➡), Granulosa Cell (➡), Thecal Cell (➡) and viteline Membrane (➡).

3.2. Immunohistochemical localization of kiss1 in the ovary

During the early- and late-quiescence phases (January and February), moderate immunoreactions of kiss1 was observed in the cells of developing follicular layer of the oocytes-I only (Figs. 2a"', a"", b"', b"" and 3 a), which increased considerably in oocytes-I with the onset of oogenesis during the early-recrudescence phases (March) (Figs. 2c"', c"" and 3 a). During the mid-recrudescence phase (April), kiss1 was expressed more intensely in the follicular cells of the oocytes-II (Figs. 2d"', d"" and 3 a). During the late-recrudescence phase (May), intensity of kiss1 immunoreactivity in the developing and fully grown oocytes-

III was almost similar as in the oocytes-II (Figs. 2e”’, e”” and 3 a). Here, the integrated density value (IDV) was separately calculated and presented for granulosa and thecal cells of the oocytes-III during the late-recrudescence phase. Once oocytes-III were fully developed, a gradual decline in kiss1 immunoreactivity in the granulosa and thecal cells of the oocytes- III was observed thereafter till spawning took place (Figs. 2f”’, f”” and 3 a). An appreciable amount of immunoreactions was also noticed in the ovulated egg during the spawning phase (July) (Figs. 2g”’, g”” and 3 a). Immunoreactivity was absent in pre-adsorbed control (Fig. 2h’ & h”). The positive controls, (processed in parallel using zebrafish ovarian sections) showed distinct immunoreactions in follicular layers (granulosa and theca cells) of the ovarian follicles (Fig. 13 a’ & a”).

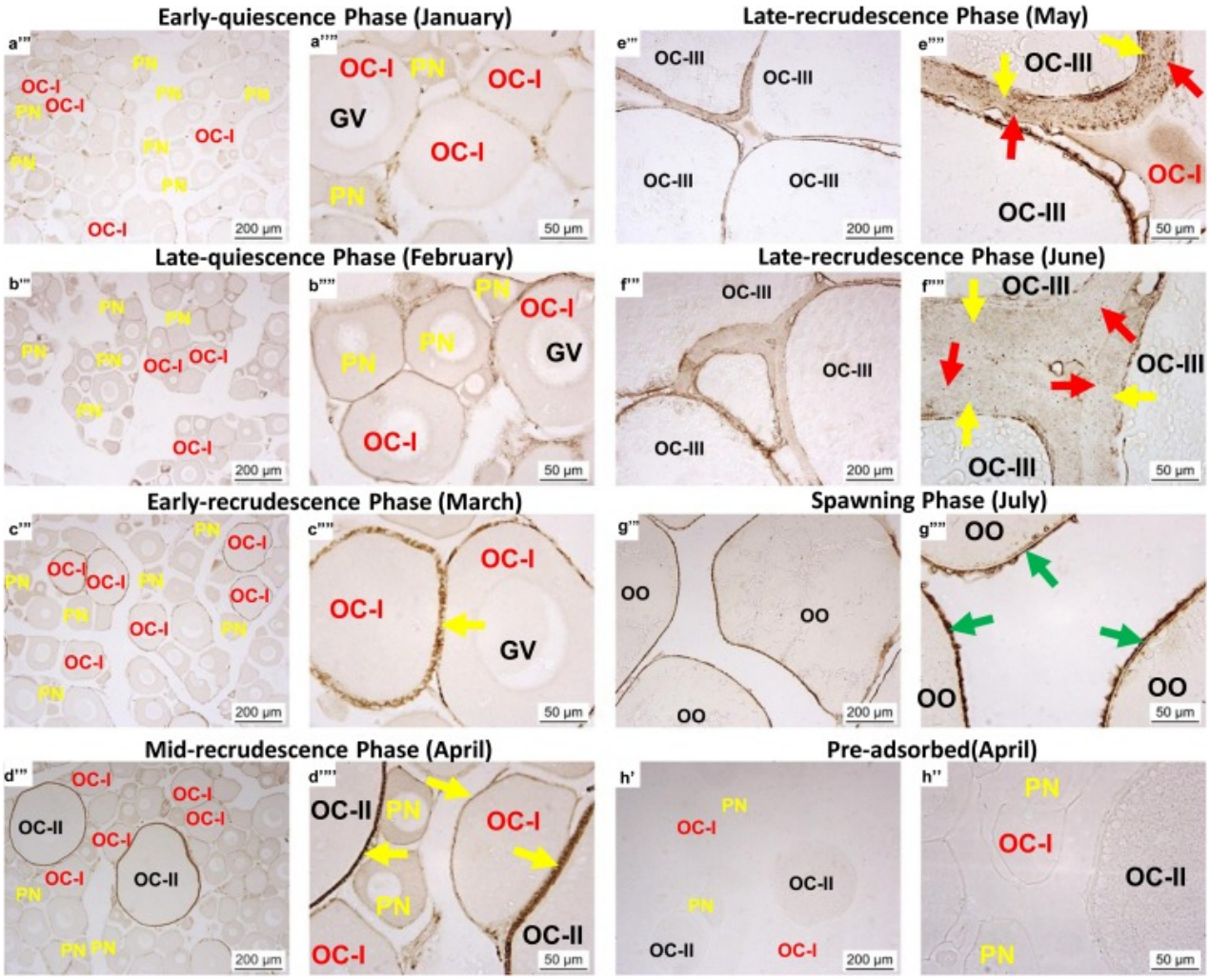

Fig. 2. Representative images of immunolocalization of kiss1 in the ovary of *Clarias batrachus* collected during the different reproductive phases, January to July (a”’ to g”’ and a”” to g””). Early−quiescence phase (January) is represented as a”’, a”” while superscripts b”’,b””; c”’,c””; d”’,d””; e”’,e””; f”’,f””; g”’,g”” are used for late−quiescence (February), early−recrudescence (March), mid-recrudescence (April), late-recrudescence (May), late-recrudescence (June), spawning (July), respectively.The pre-adsorbed control ovarian sections (h’ and h”). Note- Perinucleolar oocytes (PN), Oocytes-I (OC-I), Oocytes-II (OC-II), Oocytes-III (OC-III), Ovulated Oocytes (OO), Germinal vesicle (GV), Granulosa Cell (→), Thecal Cell (→), and Viteline Membrane (→).

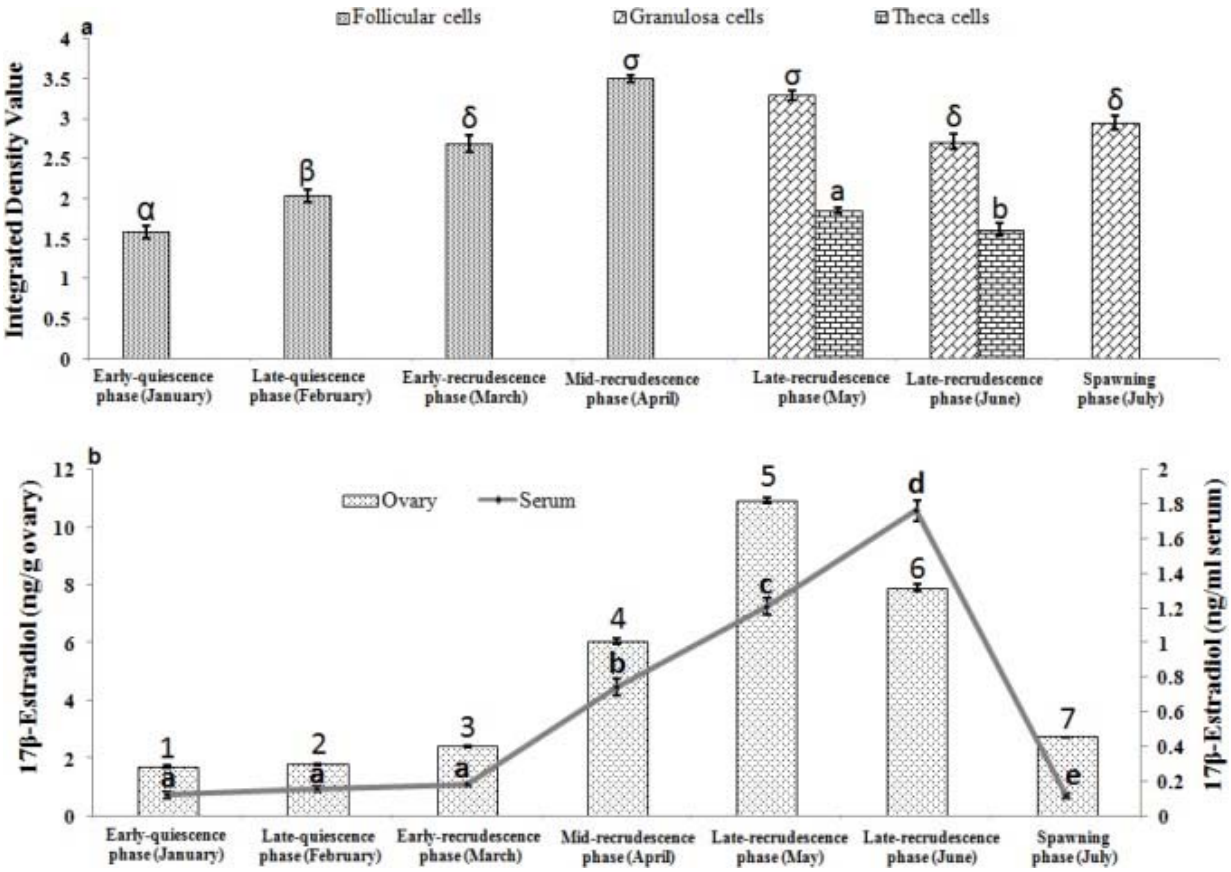

Fig. 3. IDV analysis of kiss1 immunoreaction in ovary (a) and seasonal variations in the level of 17β-estradiol in ovary and serum (b) of *Clarias batrachus* during different phases of the reproductive cycle. Each bar represents Mean±SEM (n=5). Means bearing same superscript do not differ from each other, while means bearing different superscripts are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts α, β, *δ*, & σ are used for kiss1 immunoreactivity in granulosa cells whereas, superscripts a and b for thecal cells. Superscripts a, b, c, d, & e are used for 17β−estradiol level in serum and 1, 2, 3, 4, 5, 6 & 7 for 17β−estradiol in ovarian tissue.

3.3. 17β-estradiol level in ovary and serum

Ovarian 17β-estradiol was at the basal level during the early- and late-quiescence phases (January and February). Thereafter, the levels started increasing gradually from the early-recrudescence phases (March), and reached to its maximal in the late-recrudescence phase (May). During the spawning phase (July), a sharp decline was noticed in the ovarian 17β-estradiol level (Fig. 3b). The circulating level of 17β-estradiol in the female catfish also showed similar pattern of change during different phases of the reproductive cycle (Fig. 3b).

3.4. In vivo effects of p234 on ovarian architecture

The control ovary had substantial number of all oocytes in the order of perinucleolar oocytes, oocyte-I, and developing oocytes-II. Few developing oocytes-III were also seen (Figs. 4a', a" and 5 a). However, p234 treated ovary showed predominantly early-stage oocytes (perinucleolar and oocytes-I) suggesting the suppression of folliculogenesis. The ovary of the low-dose treated catfish, revealed almost similar architecture as that of the control catfish, but no developing oocytes-III were seen (Figs. 4b', b" and 5 a). While the ovary of high-dose

treated catfish exhibited primarily early-stage oocytes (perinucleolar oocytes & oocyte-I) with a very few developing oocytes-II, significantly less than that of the control ovary. No oocyte-III was seen in the ovary of high-dose treated catfish (Figs. 4c', c" and 5 a).

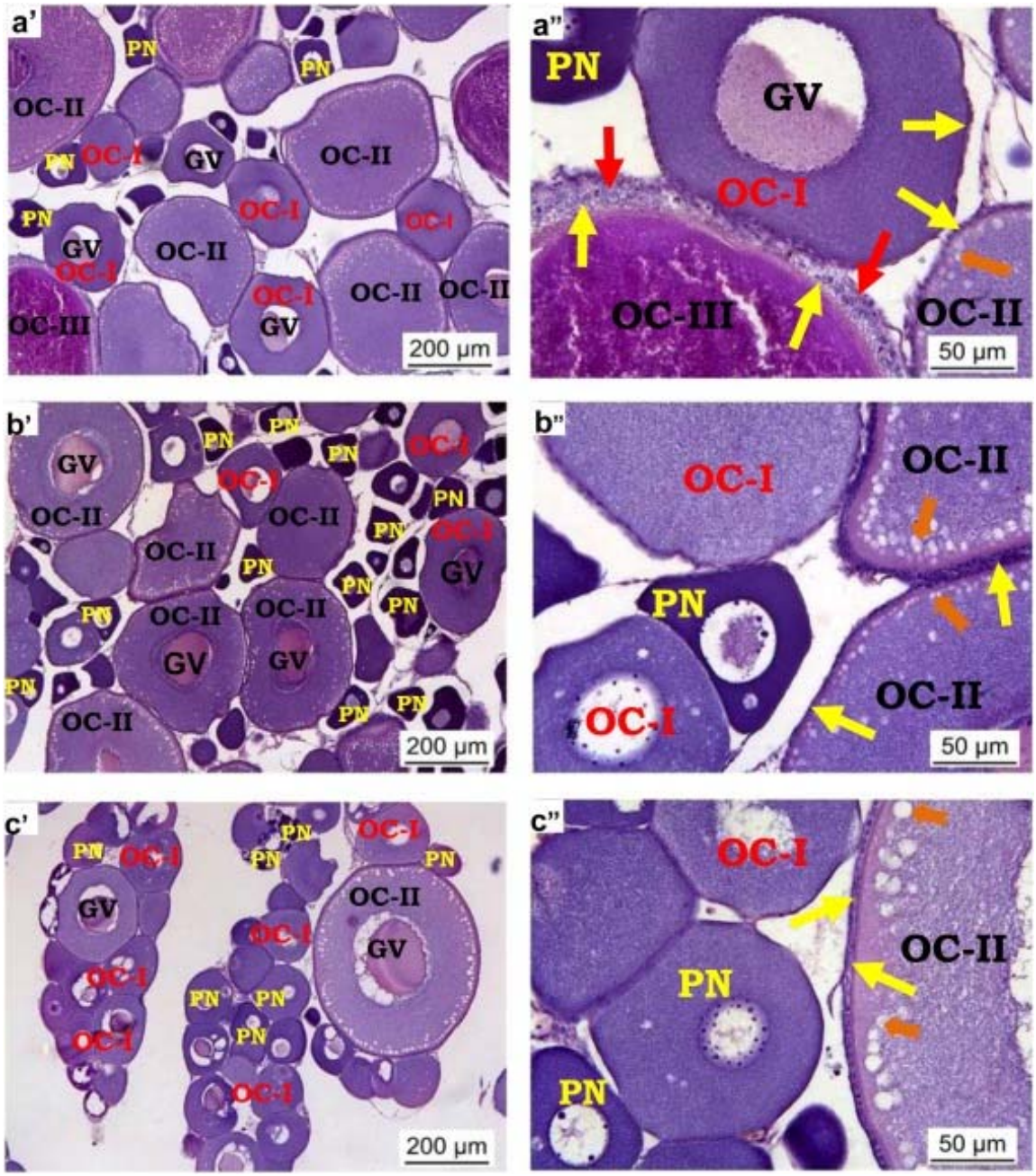

Fig. 4. Representative images of hematoxylin/eosin stained transverse sections of ovary after treatment with p234 (0.5 and 3 µg/100 g). Images were captured using Leica Microscope a' to c' and a" to c"). Fig. a' and a" represent control ovary treated with fish saline, while b' and b" are 0.5 µg/100 g treated ovarian sections. The ovary treated with 3 µg/100 gms is presented as c' and c". Note- Perinucleolar oocytes (PN), Oocytes-I (OC-I), Oocytes-II (OC-II), Oocytes-III (OC-III), Germinal vesicle (GV), Cortical Alveoli (➡), Granulosa Cell (➡) and Thecal Cell (➡).

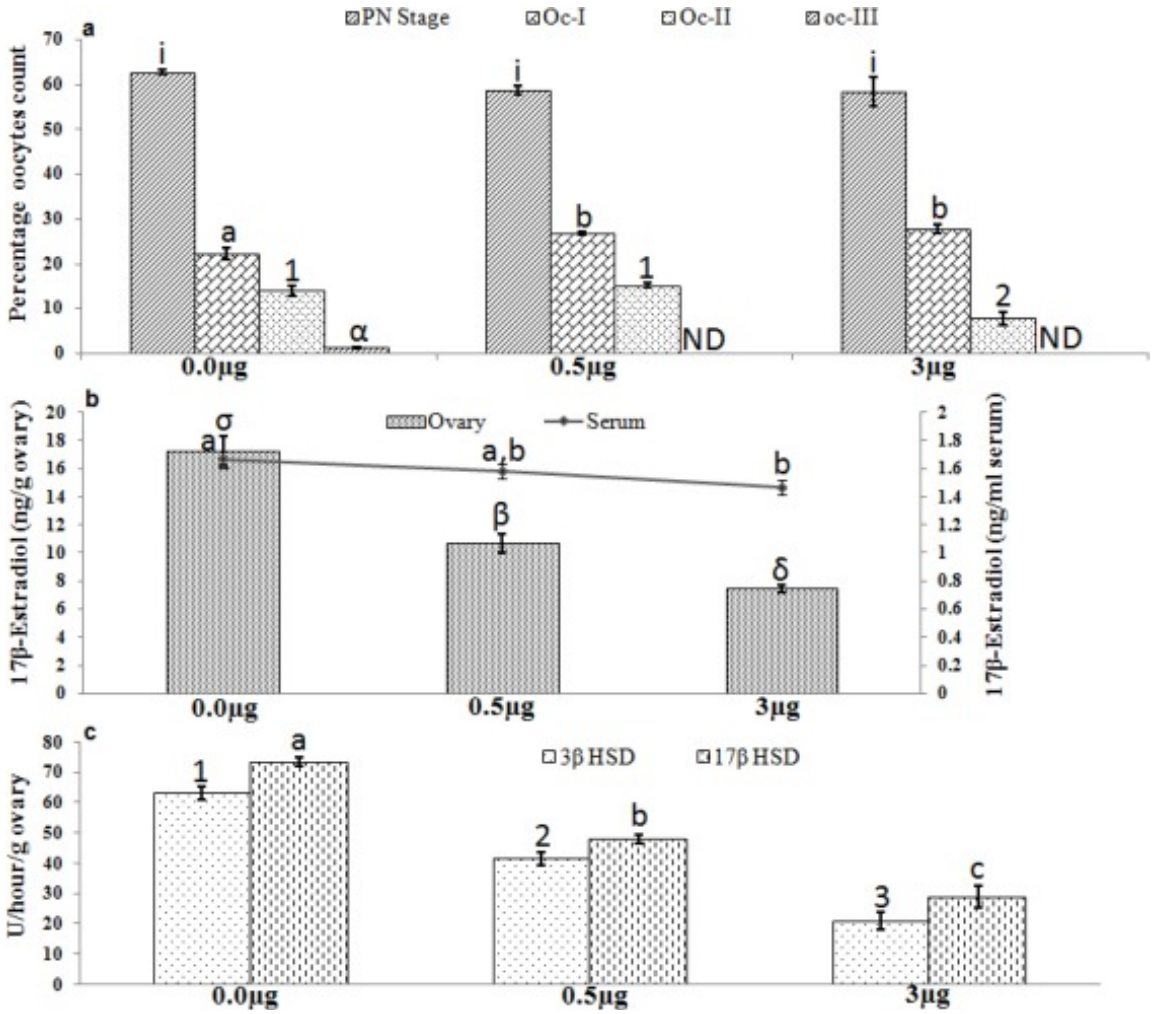

Fig. 5. Percentage oocyte count (a), ovarian and serum levels of 17β-Estradiol (b) and steroidogenic enyme activity 3β-HSD and 17β-HSD (c) in female *Clarias batrachus* after treatment with p234 (0.5 and 3µg/100g). Each bar represents Mean±SEM (n=5). Means with same superscript do not differ from each other while means bearing different superscript are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts i, a, b, 1, 2 & α are used for percentage oocytes count. Note: non detected (ND) is used for the absence of oocyte−III. Superscripts σ, β & *δ* and a & b are used for 17β−Estradiol in ovarian tissue and serum, respectively. Superscripts 1, 2 & 3 and a, b & c are used for 3β−HSD and 17β−HSD enyme activities in ovary, respectively.

3.5. In vivo effects of p234 on the level of 17β-estradiol and activities of steroidogenic enzymes in ovary

During the mid-recrudescence phase, p234 treatment decreased the levels of ovarian and serum 17β-estradiol in a dose-dependent manner (Fig. 5b). Kiss1r antagonist treatment also suppressed the activities of 3β-HSD and 17β-HSD in the ovary in the similar manner (Fig. 5c).

3.6. In vitro effects of p234 on 17β-estradiol in ovarian explants and culture medium

The p234 treatment reduced the level of 17β-estradiol in the ovarian explants as well as in the medium in dose a dependent manner (Fig. 6).

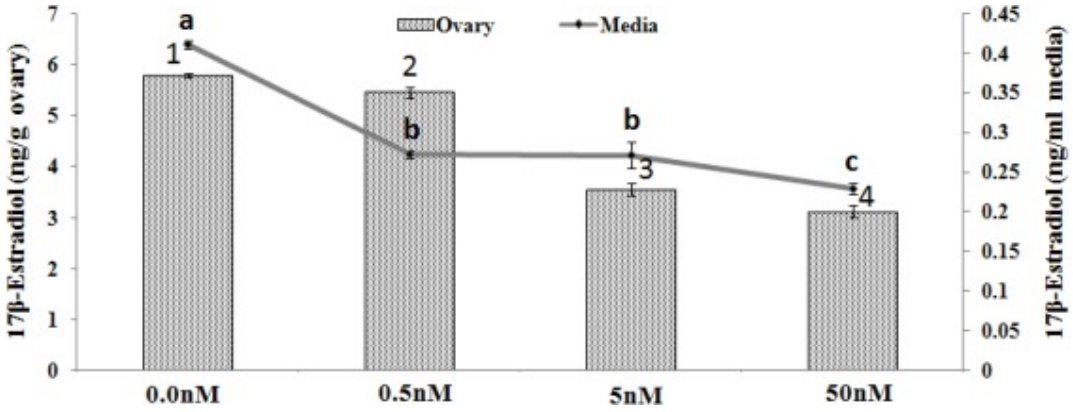

Fig. 6. *In vitro* effect of 0.5, 5, 50nM p234 on 17β−Estradiol in ovarian explants and culture medium199. Each bar represents Mean±SEM (n=3). Means with same superscript do not differ from each other while means bearing different superscript are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts 1, 2, 3 and 4 are used 17β−Estradiol in ovarian tissue and superscripts a, b and c are used for medium199.

3.7. Seasonal changes in testicular morphology

During January-February, the quiescent testis exhibited small sized seminiferous tubules and poorly developed interstitium. The germinal epithelium of seminiferous tubules was lined by Sertoli cells and well distinct spermatogonial stem cells. Lumen of the seminiferous tubules was filled with spermatogonial cells within the cysts (Fig. 7a', a"& b', b"). During the early-recrudescence phase (March), testis showed commencement of spermatogenesis with larger seminiferous tubules. Number of cysts with dividing spermatogonial cells in the seminiferous tubules was increased. Spermatogonial/ germ cells in the different cysts were found at varying developmental stages in the seminiferous tubules. Development of the interstitium had also initiated (Fig. 7c', c"). During the mid-recrudescence phase (April), the testis enlarged further and the seminiferous tubules showed more advanced germ cells ofdifferent stages in cysts as the size of the germ cells became smaller due to successive proliferation/differentiation. The interstitium developed further with distinct interstitial cells (Fig. 7d', d"). In late-recrudescence phase (May-June), testis showed well developed interstitium with large number of interstitial cells. Seminiferous tubules were filled with advanced stages of germ cells/spermatozoa (Fig. 7e', e"). During the spawning phase (July), seminiferous tubules were fully enlarged and majority of the seminiferous tubules started showing evacuation of spermatozoa (Fig. 7f', f").

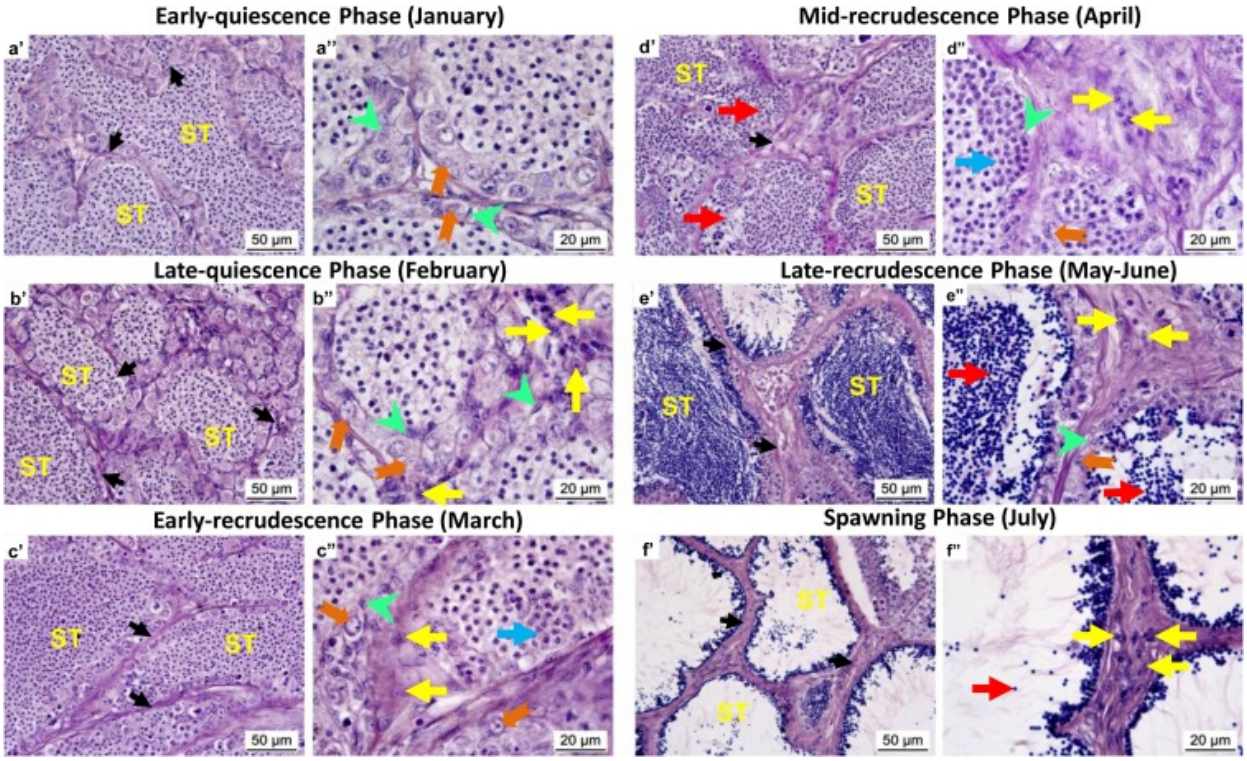

Fig. 7. Representative images of hematoxylin/eosin stained transverse sections of testis collected during the different reproductive phases, January to July (a' to f' and a" to f"). Early-quiescence phase (January) is represented as a', a", while b', b"; c', c"; d', d"; e', e"; f', f"; denotes late-quiescence (February), early-recrudescence (March), mid-recrudescence (April), late-recrudescence (May-June), spawning (July). Note- interstitium (➔), seminiferous tubule (ST), spermatogonial stem cells (➔), interstial cells (➔), advance germ cells (➔), Sertoli cells (➔), dividing cyst (➔).

3.8. Immunohistochemical localization of kiss1 in the testis

The immunoreactivity for kiss1 was observed largely in the interstitium of the testes invariably from quiescent (January) to spermeating testis (July). Immunoreactivity was very week in the interstitial cells during the early- and late-quiescence phase (Figs. 8a'", a"" & b'", b"" and 9 a). Kiss1 immunoreactivity became distinct during the early-recrudescence phase (March), and gradually increased to peak in the late-recrudescence phase (May-June) (Figs. 8 c'", c"", d'", d"", e'", e"" and 9 a). In the spermeating testis (July) kiss1 immunoreactivity decreased in the interstitial cells (Figs. 8f'", f"'& 9 a). The pre-adsorbed control sections of testis of the mid-recrudescence phase (April) (Fig. 8g', g") and late-recrudescence phase (May-June) show no immunoreaction (Fig. 8h', h"). The positive control of zebrafish testicular sections exhibited distinct immunoreactivity in the interstitial cells (Fig. 13 b', b").

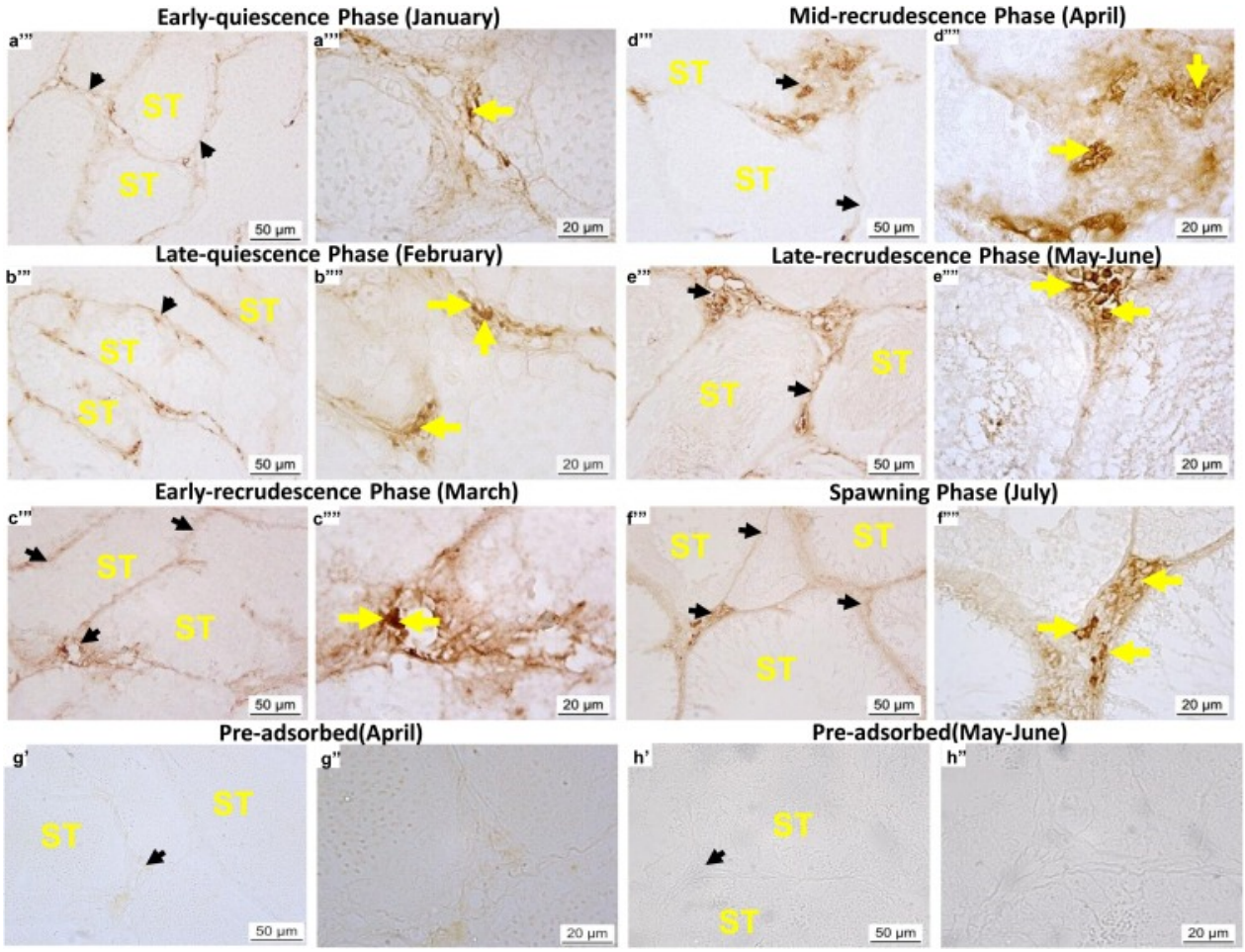

Fig. 8. Representative images of immunolocalization of kiss1 in the testis of *Clarias batrachus* collected during the different reproductive phases, January to July (a''' to f''' and a'''' to f''''). Early−quiescence phase (January) is represented as a''', a'''' while superscripts b''',b''''; c''',c''''; d''',d''''; e''',e''''; f''',f'''';'' ; denotes late−quiescence (February), early−recrudescence (March), mid−recrudescence (April), late−recrudescence (May−June), spawning (July). The pre−adsorbed control of testicular sections during mid−recrudescence (April) is represented as (g' and g'') and during late−recrudescence (May−June) as (h' and h''). Note−interstitium (➔), seminiferous tubule (ST), interstial cells (➔).

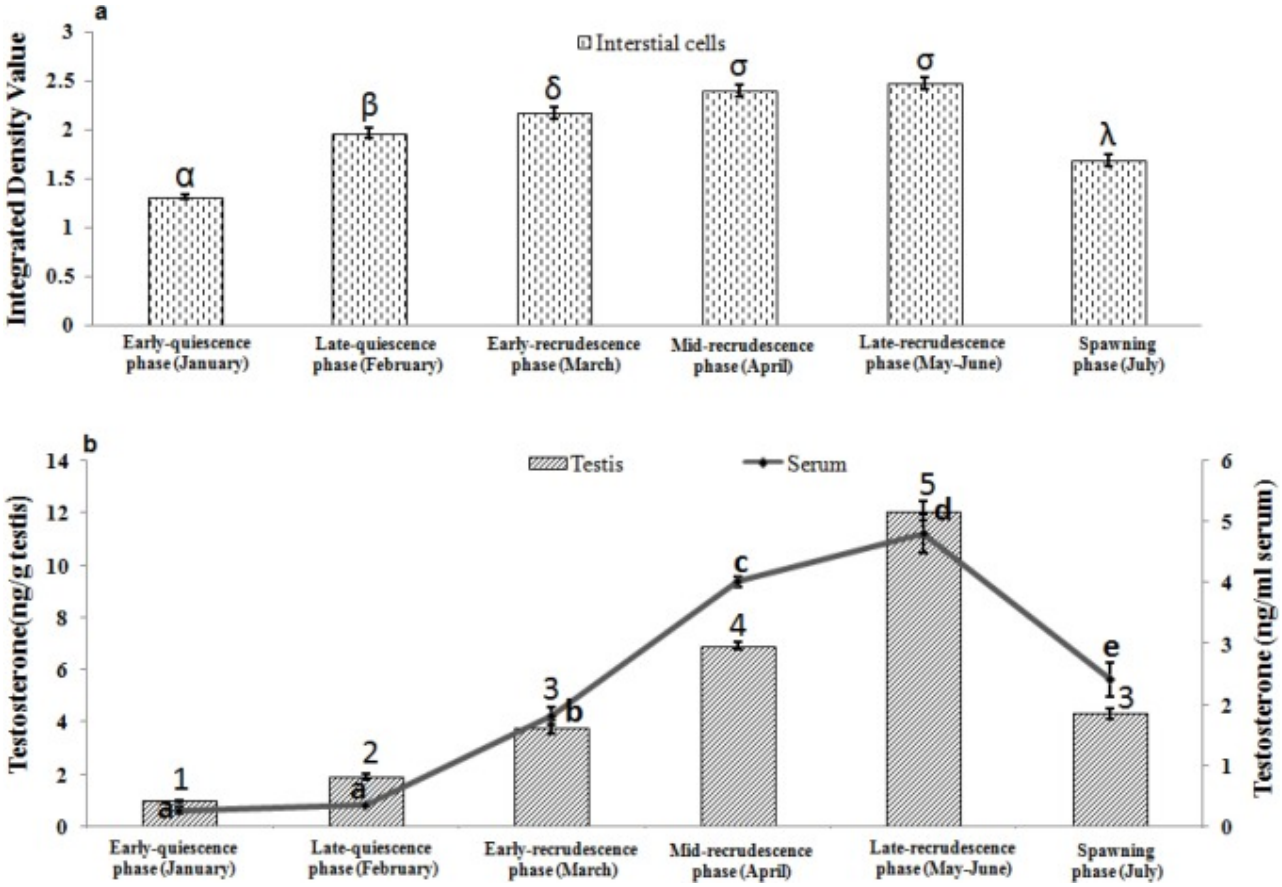

Fig. 9. Seasonal expression of kiss1 in the testis during different phases of reproductive cycle (a) and seasonal variations in testosterone concentration in testis and serum of male, *Clarias batrachus* (b). Each bar represents Mean±SEM (n=5). Means bearing same superscript do not differ from each other while means bearing different superscript are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts α, β, *δ*, σ & λ are used to compare variance among kiss1 expression and 1, 2, 3, 4 & 5 used for testicular testosterone and a, b, c, d & e for serum testosterone, respectively.

3.9. Testicular and circulating levels of testosterone

The testosterone level was low during the early- and late- quiescence phases (January-February). Testosterone level started to elevate steadily from the early-recrudescence phase (March) and reached to maximal in the late-recrudescence phase (June) (Fig. 9b). During the spawning phase (July), testosterone level dropped suddenly (Fig. 9b). Circulating level of testosterone also underwent seasonal change in similar manner (Fig. 9b).

3.10. Effects of p234 on testicular architecture

Treatment with kiss1r antagonist suppressed the spermatogenic processes, as the testis of catfish treated with low dose p234 revealed relatively low advanced germ cell/ spermatozoa and more early-stage germ cells in comparison to the control testis. The area and perimeter of the seminiferous tubules was also reduced significantly (Figs. 10b', b" and 11 a). In the high dose treated male catfish, the testis showed more early-stages germ cells (mostly spermatogonial cells). The high dose of p234 greatly reduced the development of advanced germ cells. Development of the interstitium was also arrested. In addition, area and perimeter of the seminiferous tubules were appreciably reduced (Figs. 10c', c" and 11 a).

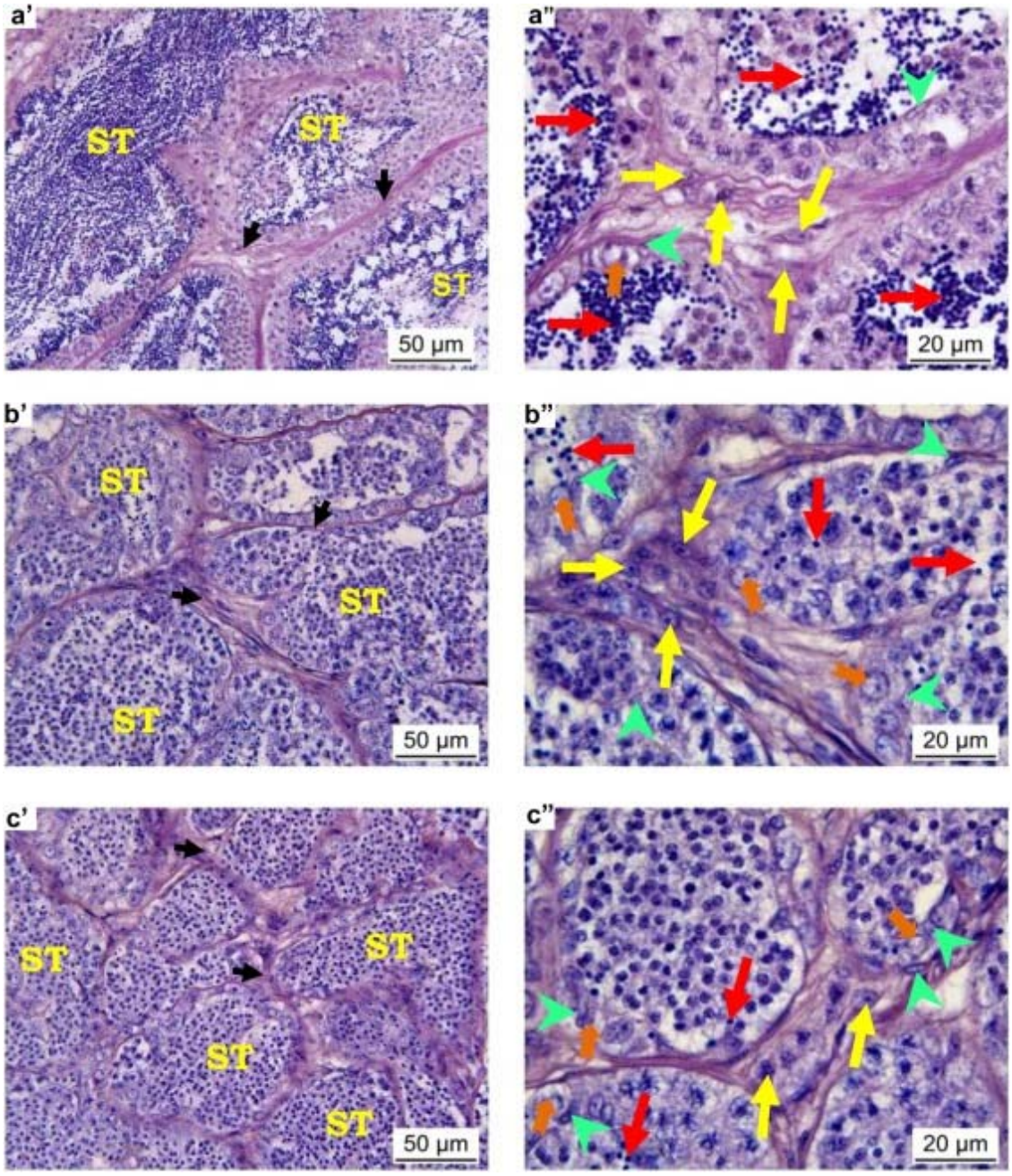

Fig. 10. Representative images of hematoxylin/eosin stained transverse sections of testis of *Clarias batrachus,* after treatment with p234 (0.0, 0.5 and 3 µg/100 g). Images were captured using Leica Microscope (a' to c' and a" to c"). Fig. a' and a" represent control testis treated with fish saline, while b' and b" are 0.5 µg/100 g treated testicular sections. The testis treated with 3 µg/100 g is presented as c' and c". Note-interstitium (➔), seminiferous tubule (ST), spermatogonial stem cells (➡), interstitial cells (➔), advance germ cells (➔), Sertoli cells (➤).

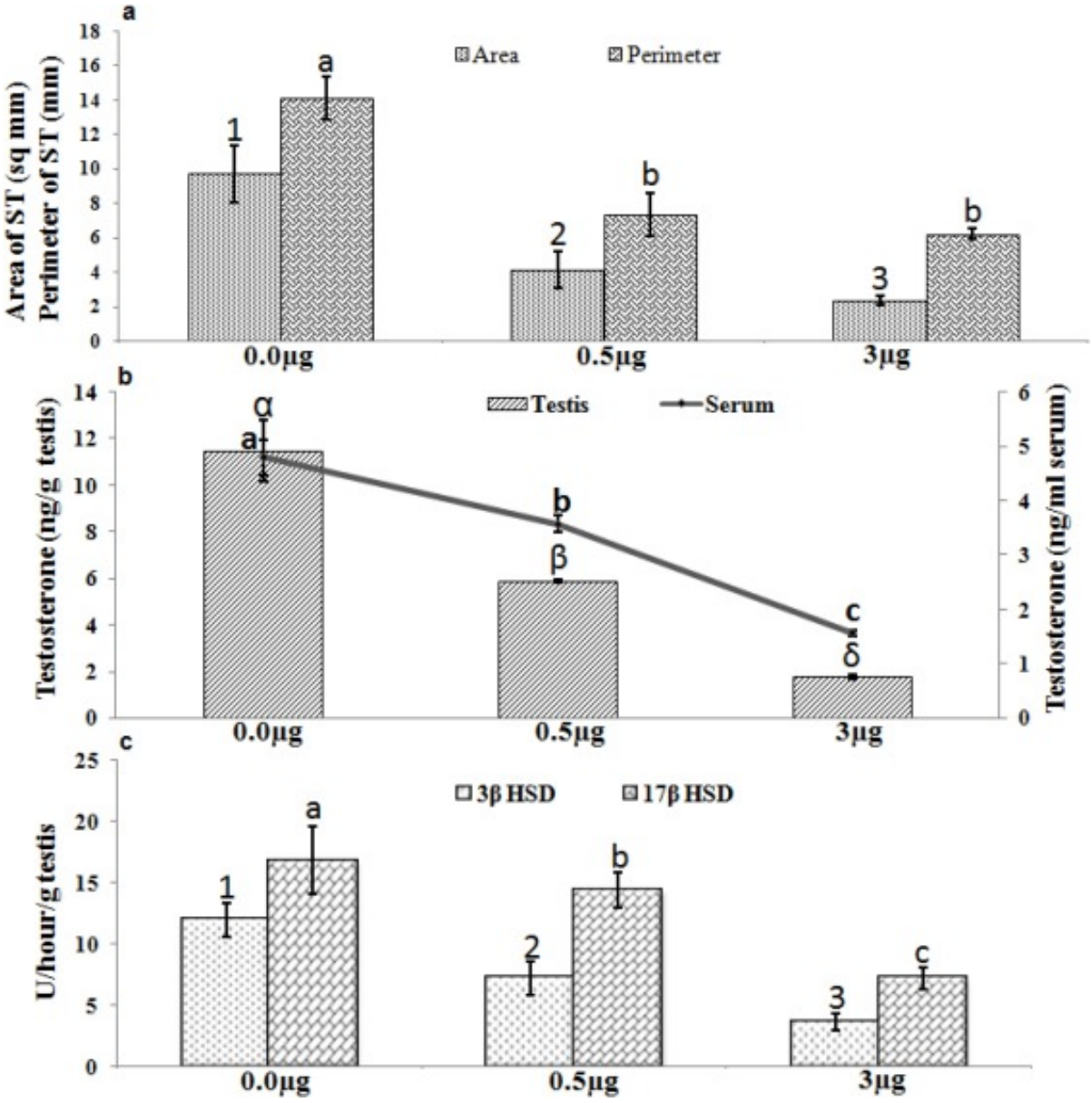

Fig. 11. Area and perimeter area of seminiferous tubules (a), testosterone concentration in testis & serum (b), and steroidogenic enyme activities of 3β-HSD and 17β-HSD (c) in male *Clarias batrachus* after treatment with p234 (0.5 and 3µg/100g). Each bar represents Mean±SEM (n=5). Means with same superscript do not differ from each other while means bearing different superscript are different from each other statistically at $P<0.05$ (Duncan's multiple range test). Superscripts 1, 2 & 3 and a & b are used to compare variance in area and perimeter, respectively. Superscripts α, β & *δ* and a, b & c are used for testosterone in testis and serum, While superscripts 1, 2 & 3 and a, b & c are used for 3β−HSD and 17β−HSD enyme activities.

3.11. In vivo effects of p234 on testosterone and the activities of steroidogenic enzyme

Kiss1r antagonist (p234) treatments decreased the concentration of testosterone in the testis and serum. The high dose of kiss1r antagonist lowered the levels of testosterone more effectively (Fig. 11b). Activities of the testicular 3β-HSD and 17β-HSD enzyme were also suppressed concurrently in a dose dependent manner (Fig.11c).

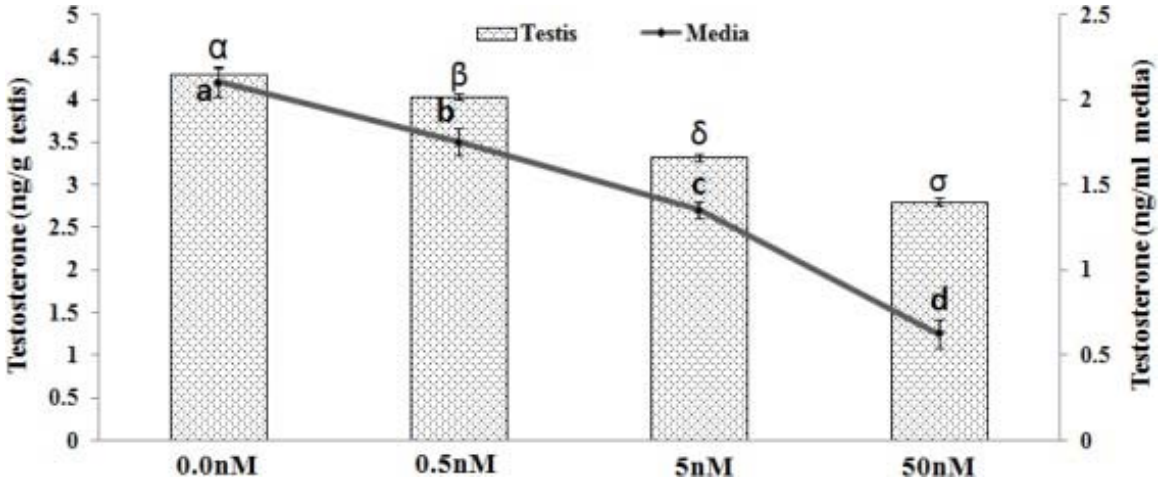

Fig. 12. *In vitro* effect of 0.5, 5, 50nM p234 on testosterone in testicular explants and culture medium 199. Each bar represents Mean±SEM (n=3). Means with same superscript do not differ from each other while means bearing different superscript are different from each other statistically at P<0.05 (Duncan's multiple range test). Superscripts α, β, *δ* and σ are used for testosterone in the testicular explants and superscripts a, b, c and d are used for medium199.

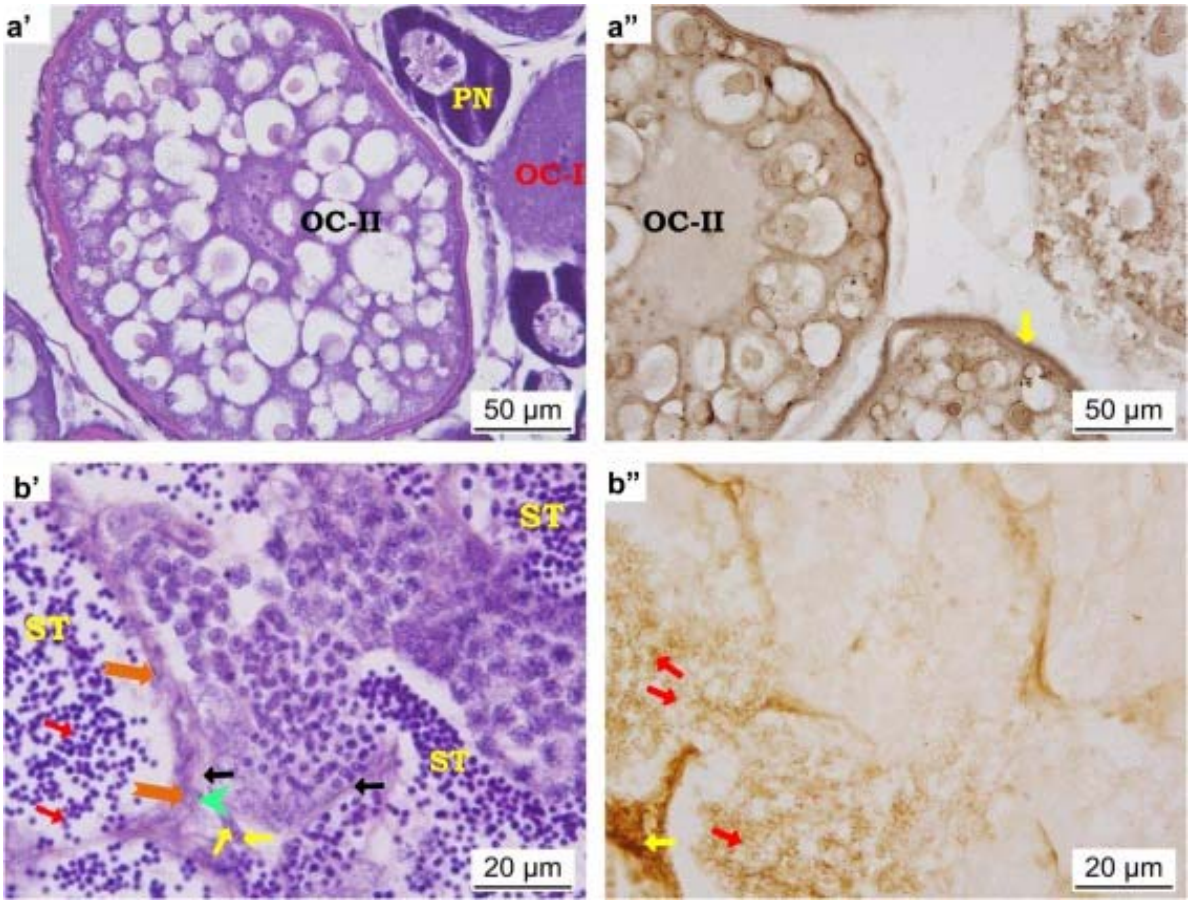

Fig. 13. Representative images of ovary (a' and a") and testis (b' and b") of Zebrafish. The Fig. a' and b' represent H&E stained sections while a" & b" show kiss1 immunohistochemistry. Note- Perinucleolar oocytes (PN),Oocytes-I (OC-I), Oocytes-II (OC-II), Granulosa Cell (➔), interstitium (➔), seminiferous tubule (ST), interstitial cells (➔), Sertoli cells (➔), advance germ cells (➔), spermatogonial stem cells (➔).

3.12. In vitro effects of p234 on testosterone in the testicular explants and culture medium

The testosterone in testicular explants and medium were also decreased in dose- dependent manner (Fig. 12).

4. Discussion

The present study reports the presence of kiss1 in different cell types of fish gonads (granulosa and theca cells of ovarian follicles, and interstitial cells in testis). The study also describes the temporal variations in kiss1 immunoreactivity in the follicular and interstitial cells, with seasonally changing ovarian and testicular activities (development, maturation and steroidogenesis) of *C. batrachus*. Study further establishes the role of kiss1 as one of the local regulators of steroidogenic and gametogenic activities in gonads of the catfish, as evident from the fact that p234, a kiss1r antagonist, decreased sex-steroid production under *in vivo* and *in vitro* conditions in a dose-dependent manner, and there by suppressed the progression of gametogenic processes.

Seasonal variations in ovarian and testicular morphology and levels of sex steroids in *C. batrachus* are in agreement with the earlier reports on seasonally breeding teleost fishes (Chakrabarti and Chatterjee, 2014; Schulz et al., 2010; Singh et al., 2008) and also recently reported for the catfish, *C.batrachus* (Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018). However, increase in serum17β-estradiol during the later part of late-recrudescence phase (June) could be because of its more release from the ovary than its production. The increased level of estradiol may be having some role in female related sexual behavior, as estradiol is known to play role in post-vitellogenic sexual behavior (Liley and Stacey, 1983). Moreover, seasonal variations in gonadal morphology and sex-steroids levels are included here as a reference for understanding of the pattern of gametogenesis in the catfish, and to correlate the expression of kisss1 peptide in different somatic and germ cell types in the ovary and testis with changing gonadal status and activities.

4.1. Kiss1 expression in the ovary

Kiss1 immunoreactivity (kiss1-ir) was observed in follicular cells and interstitial cells but the expression intensity varied with changing reproductive status of the catfish. Moderate immunoreaction of kiss1 was noted in the cells of the developing follicular layer around oocytes-I only in the quiescent ovary, which started increasing with the growth and development of the oocytes-I during the early-recrudescence phase. Kiss1 was expressed more intensely in the follicular cells of the oocytes-II and developing oocytes-III during subsequent period of folliculogenesis. Once oocytes-III was fully grown, a gradual decline in kiss1 immunoreactions in the granulosa and thecal cells of the oocytes-III was observed until ovulation and spawning. A considerable amount of kiss1-ir was also noticed in the ovulated egg. The varying degree of expression of kiss1 in the follicular cells of oocytes suggests local regulatory roles in the folliculogenic processes in fish. Its expression is not just constitutive rather appears to be physiologically relevant. Unfortunately, studies demonstrating kiss1 peptide at the cellular level in the ovary of non-mammalian vertebrates is not available, hence, it is difficult to compare the existence of kiss1-ir in follicular cells of oocytes in the catfish. However, *kiss1* mRNA and kiss1-ir have been demonstrated in several mammalian species such as in the follicular cells of ovarian oocytes of the adult rat (Castellano et al., 2006; Zhou et al., 2014), in the thecal and granulosa cells of the growing & pre-ovulatory follicles and corpus luteum of rats (Ricu et al., 2012) and cumulus-oocytes complex in mouse

ovary (Hsu et al., 2014). Recently, Cielesh et al. (2017) have shown the presence of kiss1-ir in the granulosa cells of the oocytes of all stages in the canine ovary. Further, Shahed and Young, 2009 have also reported the expression of kiss1 peptide and mRNA in the thecal and granulosa cells of the pro-estrus and estrus follicles to greater degree than in the di-estrus follicles in the seasonally breeding Siberian hamster. Thus, our results are broadly in agreement with mammalian species.

In fishes, the presence of *kiss1* has been shown in the ovary of different developmental stages but only at transcript level, without revealing the pattern of cellular distribution of *kiss1* in ovarian cells, creating difficulty in deciphering the mode and mechanism of its physiological role in regulating ovarian activity. Selvaraj et al. (2010) have shown the expression of *kiss1* mRNA, increasing gradually from the initial stage of ovarian development to the late-recrudescence phase of the ovarian cycle of chub mackerel. In immature and post-spawned *Scomber japonicas*, the level of *kiss* mRNA was very low. However, Shahi et al. (2017) have reported higher level of *kiss1* transcript during the initial stages of ovarian development than during the ovulatory period (spawning phase) in the *Tor putitora* ovary. In another study on the Indian major carp, *Labeo rohita*, the *kiss1* transcript were found to be significantly higher during the late-recrudescence and spawning phases as compared to early stages of development (Saha et al., 2016). However, Tovar Bohórquez et al. (2017) have detected very modest level of *kiss1* transcript with no seasonal change in the ovary of *Odentthestes bonarieensis*, though slightly higher *kiss* mRNA in the ovary with dominant oocytes-II (cortical alveoli stage). Recently, Bakshi and Rai, 2019 have also detected *kiss1* mRNA only in the fully grown ovary of the murrel, *Channa punctatus*. However, none of these researchers on fish has shown the presence of kiss1 peptide or transcript at a cellular level. Nevertheless, it is clear that the fish ovary does express kiss1. But the variations in its expression with changing reproductive status appear to be species-specific. The present study is unique in demonstrating the existence of kiss1 peptide at cellular level (thecal and granulosa cells) in the ovary, which suggest that kiss1 may likely be involved in steroidogenesis. However, an appreciable amount of kiss1-ir in the periphery of ovulated eggs is puzzling and needs further investigation before attributing any physiological importance to it.

4.2. Kiss1 in the testis

Like the ovary, the presence of kiss1-ir was detected in the testis of the catfish. Primarily kiss1 was recorded in the interstitium of the testis. Profiling of kiss1 in term of IDV revealed that the kiss1 signals was very week in the quiescent testis (Jan.-Feb.), but with the commencement of testicular recrudescence and steroidogenesis (March) its expression started increasing steadily to the maximal in the interstitial cells (probable Leydig cells) of fully developed testis (May-June) which decreased substantially thereafter during the spermeation (July).

Kiss1 mRNA has been detected in some fish testis by earlier workers but again not at the cellular level in the testis. Saha et al. (2016) have shown *kiss1* mRNA, invariably same in profile, in the recrudescing and spermiating testis of *L. rohita*, but was very low in the quiescent testis. Similarly, Selvaraj et al. (2010) have reported more *kiss1* transcript in spermiating chub mackerel than that of the immature or post-spawned testis. Lately, Bakshi and Rai, 2019 have also reported high *kiss1* mRNA in spermiating Indian murrel fish, *C. punctatus*. However, Shahi et al. (2017) have reported the reverse trend of *kiss1* expression in golden mahaseer, *T. putitor,* in which they have observed higher amount of *kiss1* mRNA in

the testis during initial stage of its development than that of the spermiating testis. Thus, the expression of kiss1 in the fish testis appears to be species-specific just like ovarian kiss1. The presence of kiss1 in the Leydig cells has also been reported in the testis of some mammals (Anjum et al., 2012; Irfan et al., 2016; Mei et al., 2013; Salehi et al., 2015) and amphibian (Chianese et al., 2017; Meccariello et al., 2020).

4.3. Role of kiss in gonadal activities

Based on the presence of *kiss1* mRNA in fish gonads, some authors have suggested that kiss1 might play a role in the regulation of gonadal activities in fishes, however, they have not provided any precise mode and mechanism of action of gonadal kiss1. Suggestions of most of the earlier authors rely broadly on the correlation between the profile of *kiss1* mRNA and reproductive status of the fish. While some researchers have administered kiss1 agonist and/ or antagonists *in vivo* and have opined that kiss1 helps in gametogenesis by augmenting endocrine GnRH/gonadotropin secretions from the hypothalamus and pituitary (Mechaly et al., 2013; Ohga et al., 2018; Sokołowska-Mikołajczyk et al., 2018; Zmora et al., 2015). Nevertheless, the present study for the first time provides strong evidence supporting the local action of gonadal kiss1 in gonadal activities using kiss1r antagonist *in vivo* and *in vitro* studies.

The present study reveals that kiss1r antagonist, p234, suppresses the production of estradiol in the ovary and testosterone in the testis of *C. batrachus* by lowering the activities of steroidogenic enzymes (3β-HSD and 17β-HSD) in a dose-dependent manner under both *in vivo* as well as *in vitro* conditions. Unfortunately no such study is available on fish so that the present findings could be compared.

Nevertheless, Chianese et al. (2013,2015) have proposed mutual interaction between kiss and estradiol; 17β-estradiol increases the expression of kiss1r whereas KP10 increases estrogen receptors (ER α and β) in testis of an amphibian, *Pelophylax esculentus*. Chianese et al. (2017) have also demonstrated the role of KP10 in balancing the levels of intra-testicular testosterone and estradiol in *P. esculentus*. Further, KP10 has been shown to regulate the expression of steroidogenic enzymes such as 3β-HSD, cytochrome P450, *cyp17, cyp19* at the onset of breeding cycle in *P. esculentus* (Meccariello et al., 2020). Low dose of KP10 has been observed to increase estradiol level, promoting the estradiol-dependent spermatogonial proliferation, while higher dose of KP10 is shown to stimulate testosterone production leading to testosterone-dependent activities such as spermatogenesis progression toward meiosis, sperm maturation and release in *P. esculentus* (Chianese et al., 2013; Meccariello et al., 2020).

Some studies have also been conducted in mammals to explore the role of kiss1 in gonadal steroidogenesis. Pinilla et al. (2012) have evaluated the *in vitro* effect of kiss1 on steroid production in the rat testis, where they have observed that kiss1 agonist, KP10, fails to alter the basal secretion of testosterone in rat explants, but in the monkey KP10 stimulated testicular testosterone production. Anuradha and Krishna, 2017 have shown that a low dose of KP10 stimulated progesterone production in the female bats under both *in vivo* and *in vitro* conditions, but at higher dose, it inhibited the progesterone production. Singh and Krishna, 2018 have demonstrated that KP10 treatment to PCOS ovary resulted in significant decline in testosterone level in mice at all the tested dose levels of KP10. Thus, altogether these studies on mammals suggest that kiss1 has direct action of kiss1 at gonadal levels, but it is species- and dose-specific.

The absence of studies on the effect of kiss1 on gonadal steroidogenesis in fish could be due to the fact that expression of kiss1 was not reported earlier in the steroidogenic cells in gonads, which could have triggered the investigation of role of kiss1 in gonadal steroidogenesis. Because, in mammalian species, researchers demonstrated the presence of kiss1 in steroidogenic cells (thecal, granulosa and interstitial cells), that triggered them to investigate its steroidogenic role. In the catfish, however, the demonstration of kiss1 peptide at the cellular level, particularly in the steroidogenic cells such as thecal and granulosa cells in the ovary and interstitial cells (Leydig cells) in the testis indicates the possible involvement of kiss1 in the regulation of gonadal steroidogenesis. Therefore, effect of kiss1 was evaluated on gonadal sex-steroid production under both *in vivo* as well as *in vitro* conditions. *In vitro* study was conducted particularly to explore whether kiss1 regulates steroid production in fish gonads, independent of endocrine and/ or other known regulators. The present study for the first time reports that kiss1 stimulates gonadal production of sex-steroids in fish independent of endocrine control of gonadal steroidogenesis, probably through autocrine/paracrine mechanism. Further, it can be contemplated that gonadal kiss1 may also regulate its own expression in hypothalamic kiss1 neurons through a feedback mechanism by altering sex-steroid levels in the gonads, as sex-steroid receptors have been shown to be expressed in kiss1 neurons in the hypothalamus of some fishes (Alvarado et al., 2016; Kanda et al., 2013; Mitani et al., 2010; Oka, 2009) however, such suggestion requires further experimentations.

Kiss1r antagonist, p234, treatment arrested the progression of folliculogenesis in the ovary, was evident from the prevalence of early-stage oocytes in p234 treated ovary compared to the recrudescing control ovary. Development of oocytes-III was almost completely blocked in the antagonist treated ovary. Similarly in the testis, p234 administration halted the development of advanced germ cells (spermatids/spermatozoa), which were highly dominant in the control testis. Kiss1r antagonist reduced the area and perimeter of seminiferous tubules greatly when compared with the control testis. The p234-treated testis had largely early-stage germ cells (spermatogonial cells/primary spermatocytes) suggesting thereby the arrest of spermatogenesis in male catfish. This suppression in gametogenesis in the catfish could be due to p234-induced decline in sex-steroids, as the sex-steroids are well known to regulate gametogenesis in vertebrates, including fishes (Aggarwal et al., 2014; Lamba et al., 1983; Priyadarshini Lal, 2018; Singh nee Priyadarshini and Lal, 2018; Sisneros et al., 2004) also see review (Kagawa, 2013; Lubzens et al., 2010; Schulz et al., 2010). Estrogens are established to induce synthesis of vitellogenins in liver and their release, which are then sequestered by the growing oocytes and deposited as yolk proteins in developing oocytes during oogenesis in oviparous fishes (see review Lubzens et al., 2010; Kagawa, 2013). Similarly, androgens (particularly 11-ketotestosterone) are long known to regulate spermatogenesis and other male reproductive activities in fishes (see review Schulz et al., 2010).

Surprisingly, no study has been conducted to investigate the effect of kiss1/ kiss agonist or antagonist on fish oogenesis and spermatogenesis directly. Zmora et al. (2015) has reported significant reduction in milt volume in p234-treated male *Morone saxatilis*. However, most of the authors have analyzed the impact of kiss1/kiss agonist or antagonist on GnRH/LH-FSH expression or secretion and based on the correlative studies, have opined that kiss1 stimulates GnRH/gonadotropin expression and secretion, and thereby promotes gametogenesis (Ohga et al., 2018; Sokołowska-Mikołajczyk et al., 2018), see review Mechaly et al., 2013). They have not performed any histomorphometric analyses of the ovary or testis in fish. While, some researchers have shown the expression of *kiss1* transcripts in fish gonads and have speculated about its physiological significance, but again based on correlation between the profile of kiss expression and status of gonadal development.

5. Conclusions

The present study suggests that gonadal kiss1 may act locally to regulate gonadal activities of catfish. It stimulates gonadal steroidogenesis, independent of the known endocrine and gonadal regulators, through autocrine/ and or paracrine mechanism, and by regulating steroid production, it appears to influence oogenesis and spermatogenesis.

Author statement

Ankur Singh: Conceptualization, visualization, investigation, data analysis and writing the manuscript. **Bechan Lal:** Supervision, conceptualization, reviewing and editing the manuscript. **Ishwar S. Parhar:** Kisspeptin antibodies, peptide, reviewing and editing the manuscript. **Robert P. Millar:** Kisspeptin receptor antagonist (p234), reviewing and editing the manuscript

Funding

This work was supported by the grants under the Center of Advanced Study Program phase V of the University Grants Commission (UGC), New Delhi, India, to the Department of Zoology, Banaras Hindu University, India. Ankur Singh is thankful to UGC, New Delhi, India for the award of MANF (F1 17.1/2014 15/MANF2014 15CHR UTT31,622/ (SA III/Website)).

Ethical approval

All experiment were conducted in accordance to the guidelines of Institutional Animal Ethics and Care of Banaras Hindu University, India (approval letter No. F.Sc./IAEC/2016−17/1136) and as per the Guidelines of Committee for the Purpose of Control and Supervision of experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India, New Delhi, India, for Experimentation on Fishes.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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