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The *kiss2/kiss2r* system directly modulates the activity of the pituitary gland in largemouth bass (*Micropterus salmoides*)

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ABSTRACT

The kisspeptin system, which involves hypothalamic neuropeptides, plays pivotal roles in the regulation of teleost reproduction. A previous study suggested that the *kiss2/kissr2* system in largemouth bass (*Micropterus salmoides*) was involved in regulating gonadal development through the hypothalamus-pituitary-gonad (HPG) axis. However, whether the *kiss2/kiss2r* system directly regulates the activity of pituitary gland were not studied. This study aimed to elucidate the detailed mechanism of kisspeptin-induced effects on gonadotropin hormone-producing cells in vivo and in vitro. *In situ* hybridization analysis revealed very widespread expression of *kiss2r* mRNA in the optic tectum, hypothalamus and pituitary. In the pituitary, *kiss2r*-expressing cells were mainly located in the proximal pars distalis and pars intermedia, among which the *fshβ*- and *lhβ*-positive cells exhibited expression of *kiss2r* messengers. Furthermore, primary culture of pituitary cells and peptide administration experiments revealed that the Kiss2-10 peptide stimulates the expression of *fshβ* and *lhβ* and the secretion of FSH and LH in 24 h. In addition, histology analysis revealed that three injections of the Kiss2-10 peptide promoted ovary development and increased oocyte size but had little effect on testis development. These results suggested that the intrapituitary kisspeptinergic system, as a hypophysiotropic neuropeptide factor, directly modulates of gonadotroph function. This study will help uncover the reproductive endocrinology network and improve artificial breeding technology for largemouth bass.

1. Introduction

The developmental process in animals is extremely complex and precise, and the kisspeptin system, which involves hypothalamic neuropeptides, plays a critical role in the regulation of reproduction. In the classical mammalian model, kisspeptin ligand (KISS1) produced in the brain acts via the KISS1 receptor (or G protein-coupled receptor 54, GPR54) to stimulate gonadotropin-releasing hormone (GnRH) secretion by GnRH neurons in the hypothalamus, which in turn stimulates the synthesis and secretion of gonadotropins (Follicle stimulating hormone, FSH, and Luteinizing hormone, LH) by the pituitary gland [1,2]. Subsequent studies in both diverse mammalian and nonmammalian species have confirmed that kisspeptin is an essential gatekeepers of proper reproductive maturation and function, including sexual differentiation, puberty onset, photoperiodic reproduction, fertility, energy balance, and neuroendocrine control of gonadotropin secretion and its gating by metabolic and seasonal cues [3–5].

In contrast to mammals, teleosts are known to have undergone a third genome duplication event and subsequent gene loss, and two paralogous kisspeptin genes (*kiss1* and *kiss2*) and receptors (*kissr2* and *kissr3*) have been isolated in many fish species [6–9]. In recent decades, kisspeptin-mediated regulation of reproduction via stimulation of the hypothalamic-pituitary-gonad (HPG) axis has been well documented. *In situ* hybridization and immunohistochemical approaches have revealed that kisspeptin systems are mainly located in the brain, and GnRH (GnRH1 or GnRH3) neurons expressing *kissr* mRNA have been identified in many fishes including cichlids (*Astatotilapia burtoni*), striped bass (*Morone saxatilis*), European seabass (*Dicentrarchus labrax*), chub mackerel (*Scomber japonicus*), and sablefish (*Anoplopoma fimbria*) [4, 9–12]. Furthermore, there is a growing body of work showing that the expression profiles of *kiss1/kiss3r* and *kiss2/kissr2* vary with gonadal development and are species-specific [13–16]. In addition, numerous

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studies have indicated that exogenous kisspeptin peptides (such as Kiss1-10 and Kiss2-10 peptides) stimulate the expression of HPG axis genes (brain *gnrh1* and *gnrh3*, pituitary *fshβ* and *lhβ*, and gonad *ar* and *er*), enhance the release of plasma steroid hormones, and accelerate gonadal development and maturation [16–20]. These observations accordingly provide convincing evidence that kisspeptins are positive regulators of fish reproduction via the HPG axis.

Kisspeptin directly stimulates pituitary hormone release in some mammals, including rats, cows, pigs, baboons, and sheep, and these studies revealed that the stimulatory effect of kisspeptin (Kp1-10) on LH was dose- and time-dependent and enhanced the hormonal responses to their major regulators (GnRH) without affecting the release of FSH [21–23]. Recently, studies have also described a direct stimulation of LH and/or FSH secretion from pituitary cells in response to kisspeptin in several fish. However, whether kisspeptin directly affects pituitary hormone release is controversial in teleosts. In zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*), exogenous Kp2-10 peptide strongly stimulated pituitary *fshβ* and *lhβ* expression [7,19]. In European eel (*Anguilla anguilla*), exogenous Kp1-10 and Kp2-10 peptides were able to specifically and dose-dependently inhibit *lhβ* expression, but without any effect on *fshβ* expression by primary culture pituitary cell analysis [24]. In the European sea bass, pituitary cells exhibited *kiss1* mRNA expression in most *fshβ*-positive cells and never in *lhβ*-positive cells [12].

Largemouth bass (*Micropterus salmoides*, LMB) is an economically important freshwater fish in China. Specifically, LMB is a semi-synchronous reproducer with female fish typically containing oocytes at several developmental stages, which results in a relatively long spawning period during the reproductive season [25]. For elucidation of the regulatory mechanism of gonadal development of LMB, the complete coding sequence and seasonal expression of several genes in the HPG axis, including *kiss1/kiss1r*, *kiss2/kissr2*, *gnrh3*, and gonadotropin subunits (*fshβ*, *lhβ*, *gthα*) were analyzed by searching the whole-genome sequence data and RACE sequences in previous studies [16,26,27]. Furthermore, qPCR results showed that the expression profiles of *kiss2* and *kiss2r*, but not *kiss1* and *kiss1r*, varied with the gonad development. In addition, the results demonstrated that the *kiss2/kiss2r* system stimulates the expression of HPG axis genes and accelerates the onset of puberty in LMB [16]. However, whether the *kiss2/kiss2r* system directly regulates the activity of the pituitary gland has not been studied. In this study, *in situ* hybridization, Kiss2-10 peptide administration and primary culture of pituitary cells were used to evaluate the effect of *kiss2/kiss2r* on the activity of the pituitary gland. The findings of this study may enhance our current understanding of the role of the kisspeptin system in regulating the HPG axis in semi-synchronous teleost.

2. Materials and methods

2.1. Animals and housing

In September 2022, prepubertal largemouth bass (n = 400; 5 months old, body weight = 273.62 ± 20.58 g, body length = 24.28 ± 2.55 cm) were obtained from a commercial fish farm and then reared in tanks at the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha, China. Healthy fish were maintained in tanks supplied with natural water (22.5 ± 2.3 °C) and ambient light, suitable pH (6.0–8.0), and dissolved oxygen content (5.5–7.0 mg/L). All experimental fish were fed daily to satiety with commercial pellet feed at 0.5% of the initial body weight at 12:00.

2.2. The i.p. injection of Kiss2-10 peptide

The core peptide of LMB Kiss2 (Kiss2-10: FNFNPFGLRF-NH2) was synthesized by ChinaPeptides (Shanghai, China) with a purity >95%. Prior to use, the synthetic peptide was diluted in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA).

One hundred fish were randomly collected in October; 50 individuals were used as the control group, and the rest were the experimental group. All the fish were distributed into tanks (1.5 × 1.0 × 1.0 m^3), and each tank contained 10 individuals. Saline injection was given for 2 days before the Kiss2-10 peptide injection to adapt the fish to the anesthesia and injection procedure. Then, the control group fish were treated with an *i.p.* injection of saline alone, and the experimental group fish were injected with diluted Kiss2-10 peptide at a concentration of 250 ng/g body weight. The injection volume was 0.5 mL for each fish. The dosage and timing of injections were selected as described in our previous study [16,28]. Then, fish were fed after they returned to their normal state (fed at 12:00 every day). One fish was randomly selected from each tank (n = 5) for anesthesia and subsequent pituitary sampling at 0, 3, 6, 12 and 24 h. The entire sampling process was carried out on ice, and the pituitary samples were quickly frozen in liquid nitrogen for total RNA isolation. Approximately 0.8 mL of blood was extracted by caudal puncture using heparinized syringes. Plasma was later separated by centrifugation (3000×*g*, 15 min, 4 °C) and frozen at −80 °C for hormonal analysis. The remaining fish were reared and injected with the Kiss2-10 peptide at 20 days and 40 days. No mortalities associated with the treatments were recorded. At the end of the experiment (60 days), three representative ovaries and testes were collected for histology analysis. Gonad development classification and oocyte size measurement were performed as described in our previous study [16]. Brains and pituitaries from control group fish were collected, fixed in fixative solution (4% paraformaldehyde) overnight at room temperature, dehydrated, embedded in paraffin and cut transversally in series at 8 μm. All sections were mounted onto poly-L-lysine-coated slides and kept at −20 °C for *in situ* hybridization analysis.

2.3. Pituitary cell culture

A total of 48 fish were collected for primary cell culture experiments. The pituitary cells and culture medium were prepared as described in previous studies with some modifications [16,29]. Briefly, pituitary tissues were gently excised, washed with PBS in three times, and then diced into small fragments. The fragments were digested in 0.25% trypsin solutions (containing EDTA and phenol red) (Gibco, Grand Island, NY, USA) in a 37 °C water bath with shaking for 30 min. After the cell suspension had been serially filtered through 100 μm nylon, the harvested cells were resuspended in DMEM/F-12 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Cells were seeded in the wells of 12-well plates (approx. 8 × 10^5 cells/mL/well, containing 1.0 mL of DMEM/F-12 supplemented with 10% fetal bovine serum) and cultured overnight in a biochemical incubator at 25 °C and 5% CO_2. Pituitary cells from three individuals were cultured in this condition within 5 days and observed under a microscope first. Then, after 24 h in the second experiment, the culture medium was supplemented with Kiss2-10 peptide (0.1 μM) and incubation for 3, 6, 12, or 24 h based on our previous study [16]. During incubation time, cells and culture medium were separated at × 1000 g and collected for RNA expression and hormone analysis, respectively. All treatments were performed in 5 biological replicates.

2.4. In situ hybridization

In situ hybridization was performed for *kissr2*, *fshβ* and *lhβ* in the brain or pituitary as described in previous studies [11,12]. The primers for the probes are presented in Table S1. To determine whether FSH- and LH-producing cells coexpressed *kiss2r* mRNA, sense and antisense RNA probes were transcribed and labeled with carboxyfluorescein (FAM) or cyanine 3 (Cy3) (Roche Diagnostics) and T7 RNA polymerase (Roche Diagnostics) according to the manufacturer's protocol. For hybridization, sections were air dried, postfixed in 4% paraformaldehyde for 10 min, treated with 5 μg/mL proteinase K for 5 min at 37 °C, and refixed. After incubation with 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min, the sections were prehybridized at 58 °C for 2 h and

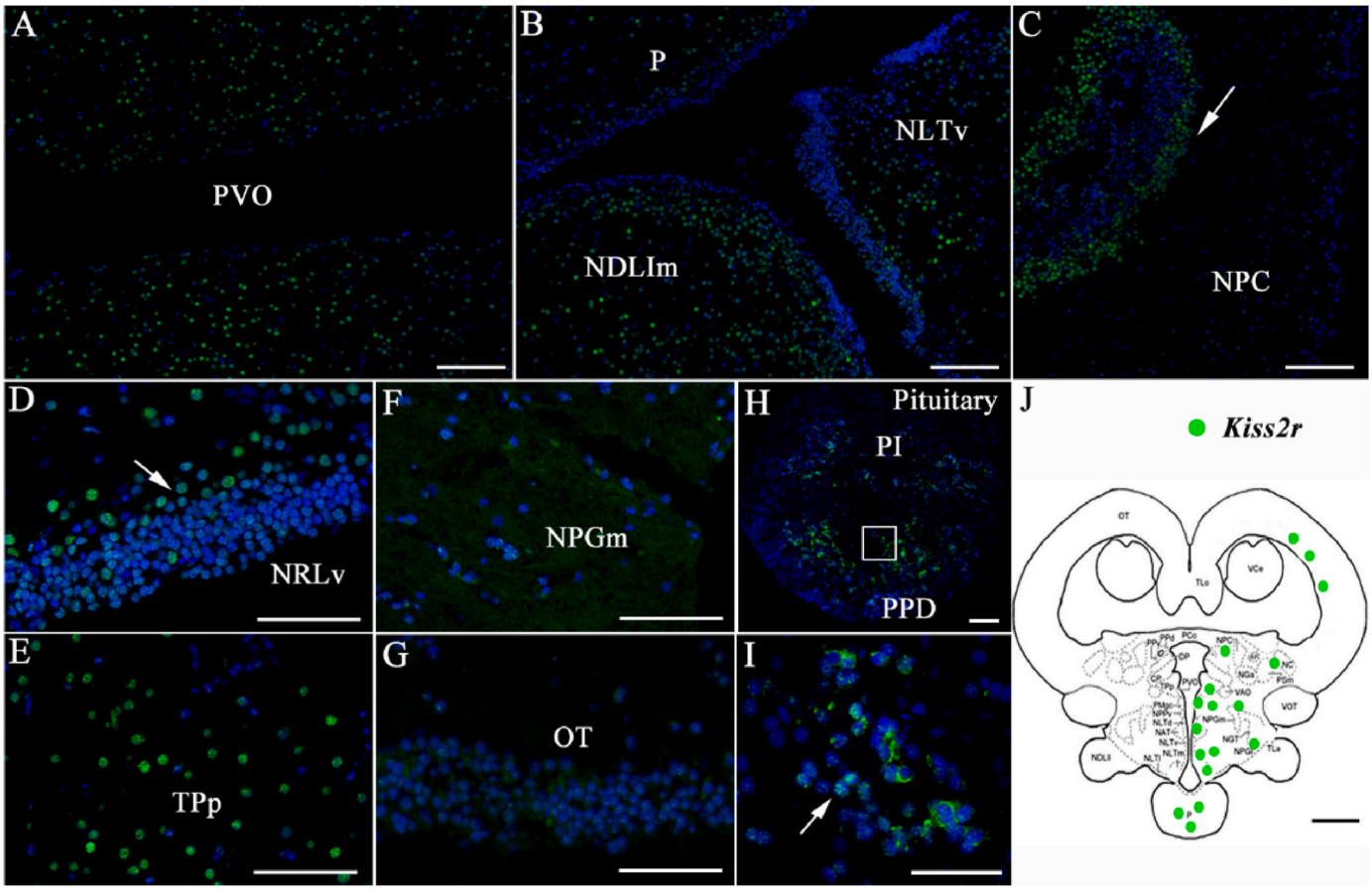

Fig. 1. Transverse sections showing the expression of *kiss2r* messengers-expressing cells in the brain and the pituitary gland of LMB. High expression of the *kiss2r* mRNA was detected in the preoptic area (PVO in A), ventral part of the lateral tuberal nucleus and medial part of the diffuse nucleus of the inferior lobe (NLTv and NDLIm in B), central pretectal nucleus (NPC in C), ventral part of the recessus lateralis nucleus (NRLv in D) and periventricular nucleus of the posterior tuberculum (TPp in E). Lower expression of *kissr2* mRNA was also observed in the medial preglomerural nucleus (NPGm in F) and optic tectum (OT in G). A strong expression of *kiss2r* was also detected in the pituitary (H), and magnification of pituitary (I). Distribution of *kiss2r* mRNAs in the brain of largemouth bass (J) and brain schematic taken from European Sea Bass [12]. A–G and I, bar = 100 μm; H: Bar = 50 μm; J: bar = 500 μm. Proximal pars distalis (PPD), pars intermedia (PI), rostral pars distalis (RPD) in the pituitary.

hybridized with the labeled riboprobe at 58 °C overnight. The probe concentrations were 500, 200, and 200 ng/mL for *kiss2r*, *fshβ* and *lhβ*, respectively. Finally, the sections were immersed in 2 μg/mL DAPI solution (Dojindo, Kumamoto, Japan) for 5 min. Double-label *in situ* hybridization was performed using a tyramide signal amplification (TSA) plus fluorescence system (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. Negative control slides were similarly treated with sense riboprobes. The fluorescence and differential interference contrast (DIC) images were observed under an LSM-700 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

2.5. Measurement of hormone levels in blood and culture medium

The levels of gonadotropins (FSH and LH) in plasma and culture medium samples were determined by enzyme-linked immunosorbent assays (ELISAs), as previously described [20,26], by using Fish FSH and LH ELISA Kits (Nan Jing Jian Cheng Bioengineering Institute, China) according to the manufacturer's protocols. The ELISA kit intra-assay and interassay coefficients of variation were less than 15%. The detection ranges are 0.75–12 IU/L for FSH and 1.5–48 mIU/mL for LH.

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissue and cell samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality of the isolated RNA was assessed by 1.5% agarose gel electrophoresis, and the concentration was measured using a multifunction microplate reader (Biotek Cytation 5, USA). Each total RNA (1 μg) sample was treated with DNase I (RQ1 RNase-free DNase) to remove genomic DNA and, then, reverse transcribed according to the manufacturer's protocol using a PrimeScript RT Reagent Kit (RR047A; TaKaRa). The primers used in this study are listed in Table S2 qPCR was performed with triplicate technical replicates in a LightCycler® 96 thermal cycler (Roche, Switzerland), with the *eef1a1* gene being used as an internal control for the normalization of gene expression. The 20 μL reaction mixtures consisted of 1.0 μL of template, 10.0 μL of SYBR Green Master Mix (TaKaRa), 1.0 μL of each of the upstream and downstream primers, and 7.0 μL of RNase-free ddH_2O. The amplification conditions were as follows: 50 °C for 5 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 60 s. After relative quantification, melting curve analysis was used to verify the generation of a single product. The average threshold cycle (Ct) was calculated for each sample using the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analysis

The data are presented as the mean ± the standard error of the mean (SEM). Gene expression and both hormonal content and plasma levels were analyzed using a two-way ANOVA (SigmaStat 3.5 SYSTAT Software Inc., Richmond, CA, USA), followed by all pairwise multiple

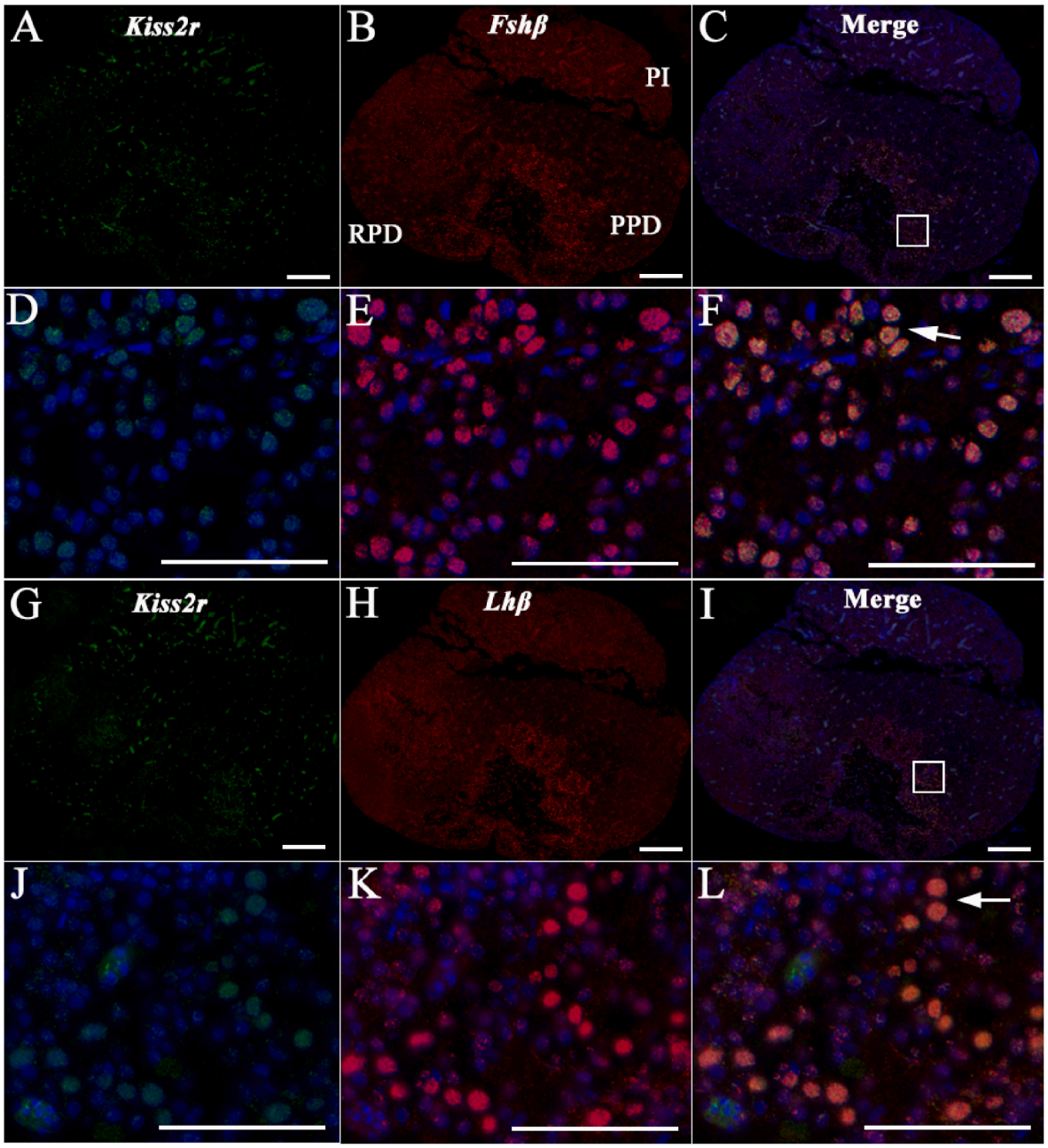

Fig. 2. Transverse sections showing the expression of gonadotropin (*fshβ* and *lhβ*) and *Kiss2r* messenger-expressing cells in the pituitary gland of LMB by *in situ* hybridization. The expression of *kiss2r* mRNA (green in A), *fshβ* mRNA (red in B) and the merged image (C) in the pituitary gland. D, E and F are magnifications of A, B and C, respectively. The expression of *kiss2r* mRNA (green in G), *lhβ* mRNA (red in H) and merged (I) in the pituitary gland. J, K and L are magnifications of G, H and I, respectively. Arrows showed the positive mRNA signal of *kissr2* and *fshβ* and *lhβ*. Proximal pars distalis (PPD), pars intermedia (PI), rostral pars distalis (RPD) in the pituitary. A–C and G–I: bar = 200 μm. D-F and J–L: bar = 50 μm.

comparison procedures (Tukey's test). Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Localization of kiss2r neurons in the brain and pituitary

Very widespread expression of *kiss2r* mRNA was identified in the optic tectum (OT), hypothalamus and pituitary (Fig. 1). Specifically, in the dorsal hypothalamus, dispersed positive cells were found in the paraventricular organ (PVO), ventral part of the lateral tuberal nucleus (NLTv), medial part of the diffuse nucleus of the inferior lobe (NDLIm), and central pretectal nucleus (NPC) (Fig. 1A–C). Moreover, a very prominent population of *kiss2r* expressing cells was identified in the ventral part of the recessus lateralis nucleus (NRLv) and periventricular nucleus of the posterior tuberculum (TPp) (Fig. 1D and E). In addition, lower expression of *kissr2* mRNA was observed in the medial preglomerural nucleus (NPGm) and optic tectum (Fig. 1F and G). In addition, a very prominent population of *kiss2r*-expressing cells was detected in the pituitary, including the proximal pars distalis (PPD) and pars intermedia (PI) (Fig. 1H and I). The distribution of *kiss2r* mRNAs in phenotypically identified neurons of the brain and pituitary is presented in Fig. 1J.

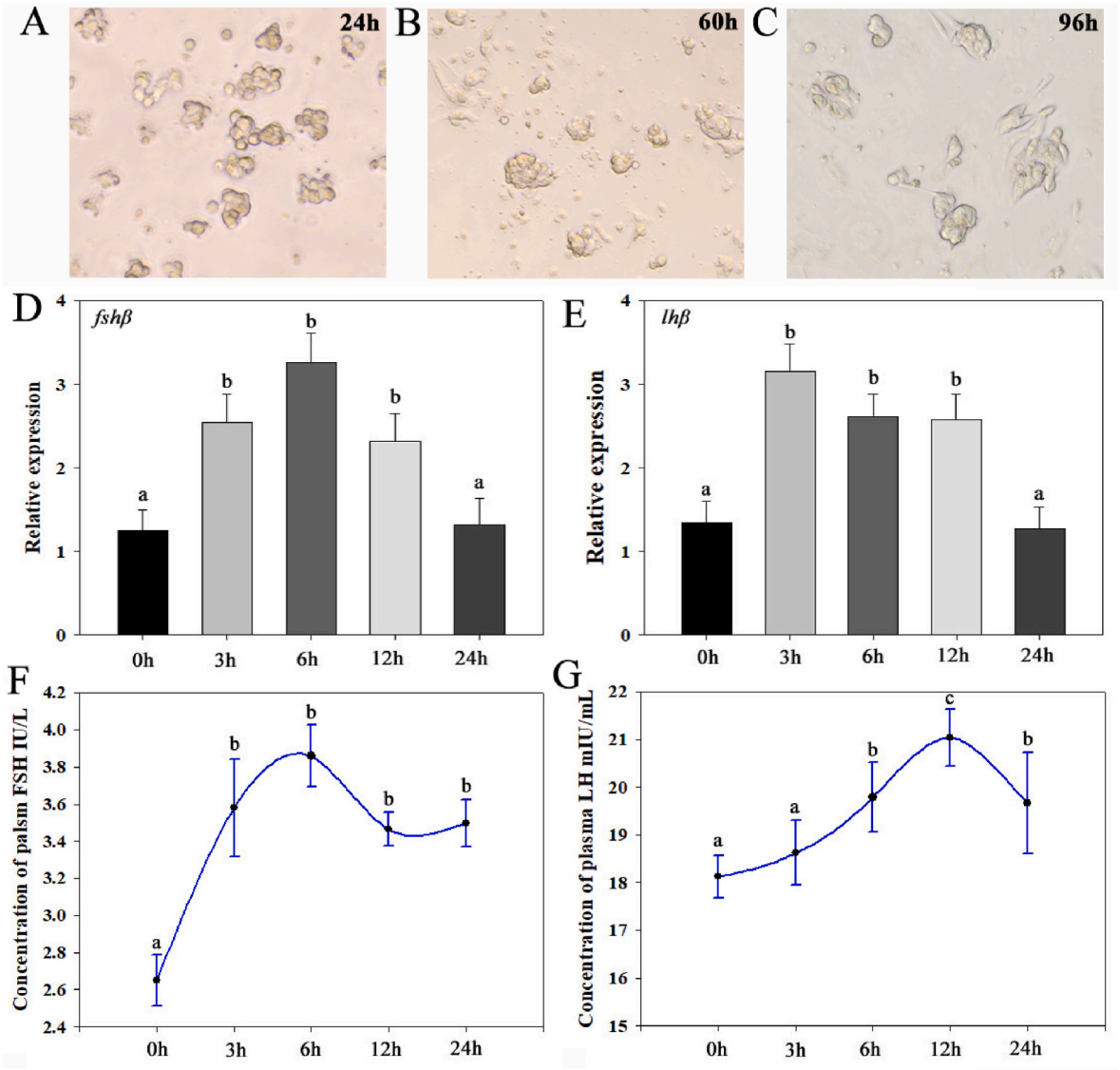

Fig. 3. Expression and activity of GTHs in LMB pituitary cell culture after Kiss2-10 peptide treatment. Pituitary cell culture and observation under a microscope at 24 h (A), 60 h (B), and 96 h (C). Changes in the expression of *fshβ* (D) and *lhβ* (E) in pituitary cells at 0, 3, 6, 12 and 24 h after Kiss2-10 peptide treatment (0.1 μM). The activity of FSH (F) and LH (G) in culture medium at 0, 3, 6, 12 and 24 h after Kiss2-10 peptide treatment. The *eef1a1* gene was used as the reference gene. Different letters represent significant differences among groups (n = 5, one-way ANOVA, followed by Duncan's test, $p < 0.05$).

3.2. Coexpression of kiss2r and gonadotropin (fshβ and lhβ) in the pituitary gland

The expression of *kiss2r* mRNA was observed to be wildly distributed in the rostral pars distalis (PPD) and RPD of the pituitary (Fig. 2A, D, G and H). Then, the expression of gonadotropin (*fshβ* and *lhβ*) and *kiss2r* messenger-expressing cells in the pituitary gland was detected. Appreciable *fshβ* mRNA was observed in the PPD and PI but not in the RPD of the pituitary (Fig. 2B and E). *lhβ*-positive cells were mainly observed in the PPD and RPD of the pituitary ((Fig. 2H and K). Coexpression was evident in some of the *fshβ*- and *lhβ*-positive cells. Almost all *fshβ*-positive cells exhibited expression of *kiss2r* messengers (Fig. 2C and F). However, many but not all *lhβ*-positive cells exhibited expression of *kiss2r* messengers (Fig. 2I and L).

3.3. Activation of gonadotropin by Kiss2-10 peptides in pituitary cell culture

LMB Pituitary cells were successfully cultured and observed under a microscope at 24 h, 60 h and 96 h (Fig. 3A–C). Then, to better understand the effect of exogenous Kiss2-10 peptide on pituitary cells, we added Kiss2-10 peptide after 24 h of incubation, and the mRNA levels of *fshβ* and *lhβ* gene and the level of FSH and LH in the culture medium were evaluated. The expression level of *fshβ* gradually increased and peaked at 6 h ($p < 0.05$), after which it gradually decreased from 12 to 24 h (Fig. 3C). The levels of *lhβ* were significantly increased and peaked at 3 h ($p < 0.05$), and then decreased from 6 h to 24 h (Fig. 3D). A similar pattern was detected with respect to FSH and LH in the cultured medium. The level of FSH exhibited a significant increase from 3 h and peaked at 6 h ($p < 0.05$), which then slightly decreased from 12 h to 24 h (Fig. 3E). However, the level of plasma LH gradually increased, peaked at 12 h and then decreased at 24 h (Fig. 3F).

3.4. Activation of gonadotropin and gonad development by Kiss2-10 peptides after injection

In response to intraperitoneal injection of exogenous Kiss2-10 peptide, the expression level of *fshβ* was significantly increased at 3 h and

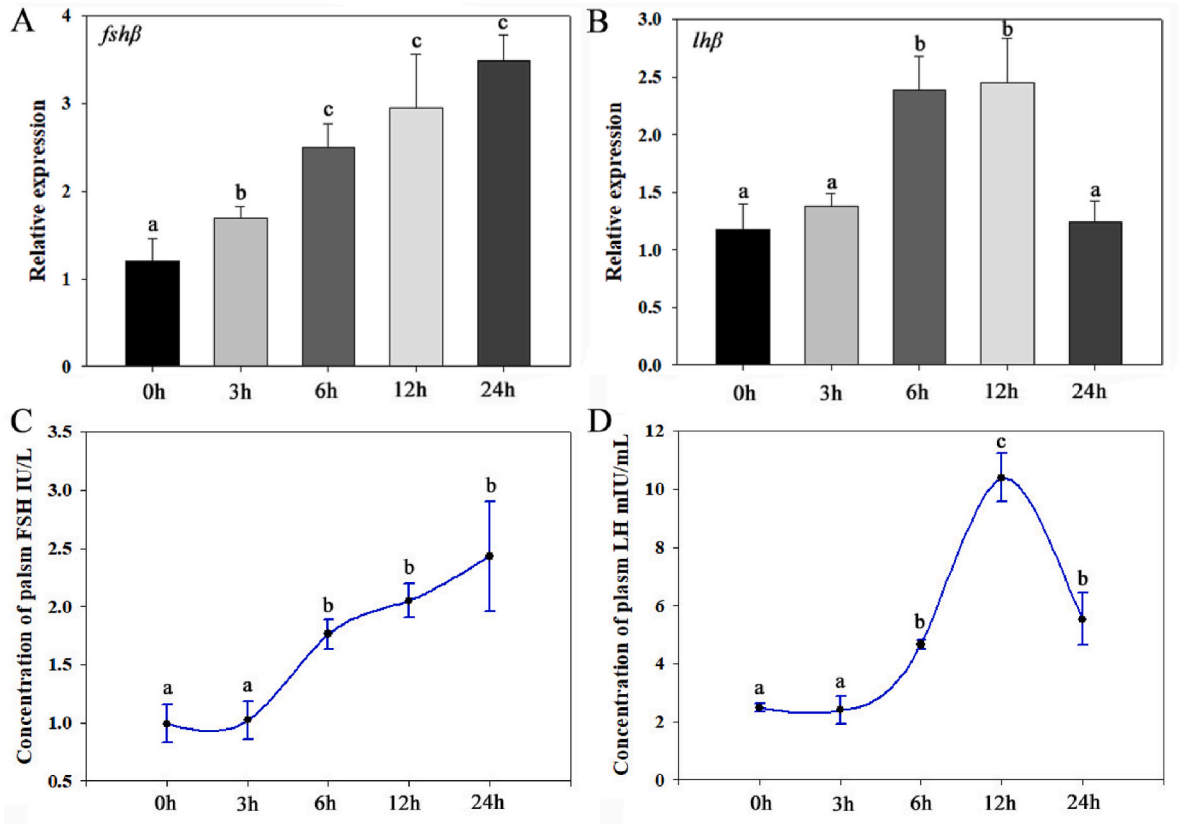

Fig. 4. Expression and activity of GTHs in LMB pituitary glands after Kiss2-10 peptide (250 ng/g) injection. Changes in the expression of *fshβ* (A) and *lhβ* (B) at 0, 3, 6, 12 and 24 h following injection of the Kiss2-10 peptide. The activity of plasma FSH (C) and LH (D) of LMB at 0, 3, 6, 12 and 24 h after Kiss2-10 peptide injection. The *eef1a1* gene was used as the reference gene. Different letters represent significant differences among groups (each group fish n = 5, one-way ANOVA, followed by Duncan's test, $p < 0.05$).

then maintained a higher expression level and peaked at 24 h ($p < 0.05$) (Fig. 4A), while the levels of *lhβ* gradually increased and peaked at 6 h ($p < 0.05$), and then decreased from 12 h to 24 h (Fig. 4B). A similar pattern was detected with respect to plasma FSH and LH. The level of FSH exhibited a significant increase and peaked at 24 h ($p < 0.05$) (Fig. 4C), while the level of plasma LH was significantly increased and peaked at 12 h, and then decreased in 24 h (Fig. 4D).

After three injections of the Kiss2-10 peptide, histology analysis revealed that the ovaries from the control group fish were classified at the primary growth cortical alveoli (PG ca) stage (Fig. 5A), while those of the peptide-treated females were classified at the early vitellogenesis (eVtg) stage (Fig. 5C). In male fish, the testes of the control group and peptide-treated fish showed no difference, and both were classified as being at the maturation stage and an abundance of mature spermatozoon was observed (Fig. 5 B and D). Moreover, the average oocyte size was larger in the Kiss2-10 peptide injection group than in the control group (Fig. 5E).

4. Discussion

The kisspeptin system, including two kisspeptins, *kiss1* and *kiss2*, and their receptors, *gpr54-1* and *gpr54-2*, is involved in the regulation of hypothalamic-pituitary-gonadal axis function in teleosts. *Kiss1* and *kiss2* are discretely distributed in the brain, with *kiss1* being highly expressed in the optic tectum-thalamus, while *kiss2* is mainly detected in the hypothalamus, telencephalon, and optic tectum-thalamus [6,7,30]. The localization of neurons expressing *Kiss2* has been reported more caudally in and around the NRL, NLT, NVT, POA and NPPv in parts of the hypothalamus and therefore serve hypophysiotropic functions [4,12,31,32]. Similarly, the two kisspeptins have also been reported in the pituitary and gonads, suggesting possible direct involvement in gonadotropin release and gonad maturation [16,32–34]. However, there is little information on the distribution of kisspeptin receptors in the brains of teleosts. Only in European seabass, chub mackerel, zebrafish and medaka, were *kiss2r* messengers-expressing cells identified as widespread in the brain, especially in the POA, NDTL, NPPv, NVT, and NRL of the hypothalamic nucleus [4,12,35]. Similar results were observed in largemouth bass (Fig. 1). In this study, *kiss2r* mRNA was widely distributed in the NLTv, NDLIm, NPC, NRLv, TPp, and NPGm of the hypothalamic nucleus, indicating a very good correlation between the wide distribution of *kiss2r*-expressing cells and Kiss2-positive fibers, which has been reported in other teleosts, suggesting that *the kiss2/kiss2r* system is vital in the neuronal network.

The distribution of kisspeptin and its receptors in the pituitary of teleosts has been examined by either *in situ* hybridization or RT-PCR studies, but only in limited species. In catfish and zebrafish, the pituitary contains a distinct group of *kiss2* cells in the proximal pars distalis (PPD) [32,33]. In European sea bass, Kiss2-immunoreactive cells are located in the pituitary PI and PPD, and colocalize with FSH- and LH-immunoreactive cells in the PPD [12,36]. These results suggested that the role of the pituitary kisspeptin system is independent, as KISS-GnRH regulation is not conserved (lack of *kissR* in GnRH neurons). In this study, we also detected *kiss2r* mRNA located in the PPD and PI of the pituitary. Our result also showed that both FSH- and LH-positive cells exhibited the expression of *kiss2r* messengers in the PPD of the pituitary (Fig. 2), indicating that at least at the early maturation stage, the *kiss2/kiss2r* system directly modulates the activity of gonadotrophs. However, the timing point and extent of the controls remain unknown and need further investigation. Notably, in zebrafish, *kiss2r* immunoreactivity was observed in corticotropes but not in gonadotropes,

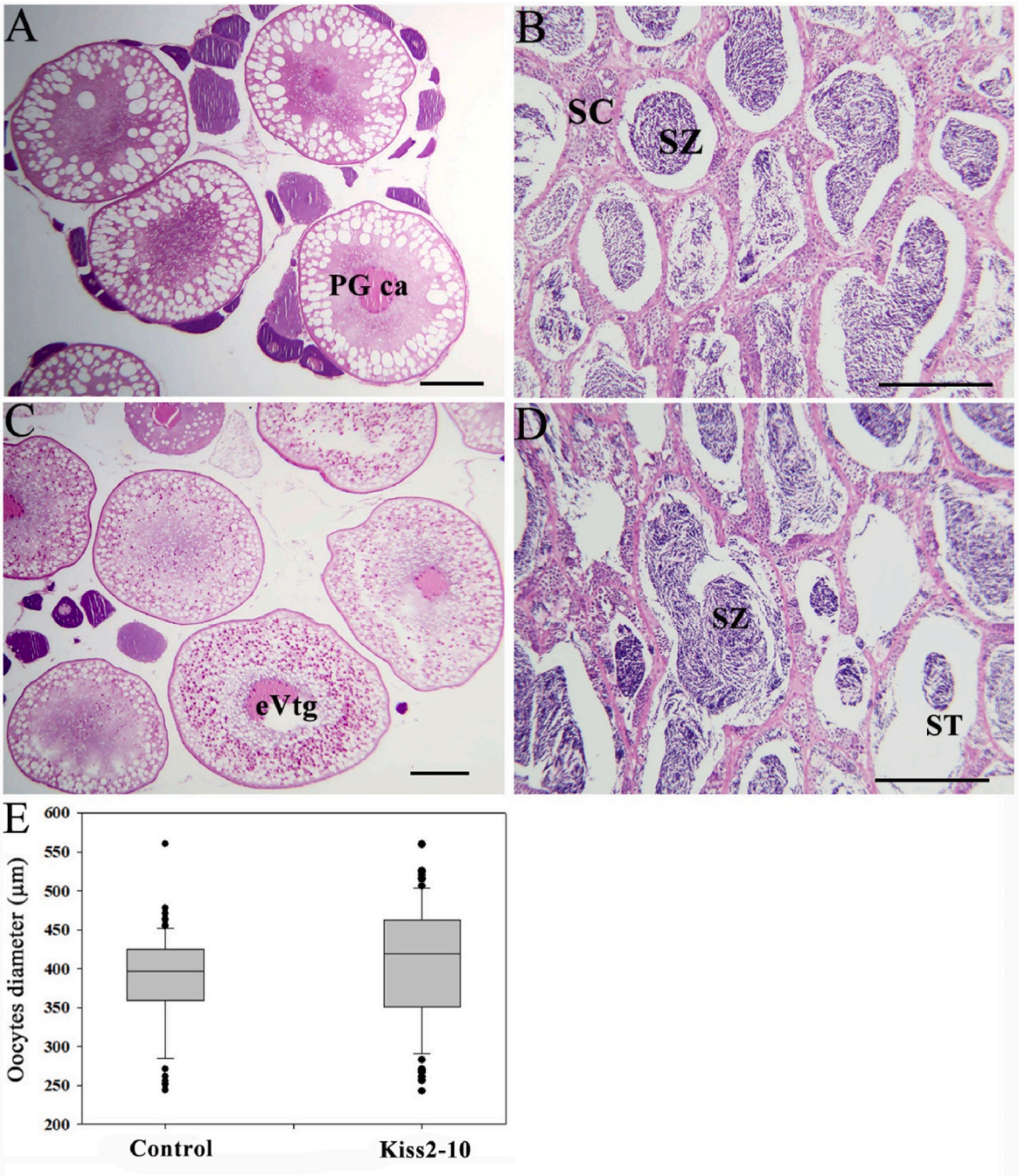

Fig. 5. Histology and oocyte size of LMBs after Kiss2-10 peptide (250 ng/g) injection. Representative ovary (A) and testis (B) microstructures of LMB in the control groups; representative ovary (C) and testis (D) microstructures of LMB in Kiss2-10 peptide injection group. E, Oocyte size of the control group and Kiss2-10 peptide injection group fish. SC, spermatocytes; ST, spermatids; SZ, spermatozoon. PG ca, primary growth cortical alveoli; eVtg, early vitellogenesis. Bar = 200 μm.

suggesting that *kiss2* and *kiss2r* signaling directly serve nonreproductive functions and indirectly subserve reproductive functions [31]. Taken together, these data in teleost fish raise the distinct possibility of intra-pituitary kisspeptinergic control of gonadotroph function, either as a hypophysiotropic neuropeptide or as an autocrine/paracrine factor.

The kisspeptin system modulates gonadotroph activity in many fishes in vivo and in vitro but is species specific. In sexually mature female zebrafish, mature male sea horse and Nile tilapia (*Oreochromis niloticus*), administration of the Kiss2 peptide strongly stimulated the pituitary *fshβ* and *lhβ* expression [7,29,37]. However, in sexually mature female orange-spotted grouper (*Epinephelus coioides*), administration of Kiss2-10 only significantly increased pituitary *fshβ* mRNA levels [38]. In European eel pituitary primary cultures, kisspeptin exerted a time- and dose-dependent inhibitory effect on *lhβ* expression [39]. In striped bass and European sea bass pituitary primary cultures, the Kiss2 peptide stimulated FSH and LH expression and secretion [18,36]. In sexually mature female goldfish, administration of Kiss1-10 stimulated serum LH levels but failed to influence LH release from pituitary cells in vitro [6]. The present study shows that LMB *kiss2/kiss2r* system activated of gonadotropin subunit genes expression and stimulated both FSH and LH release in vivo and in vitro, which are important for hormone feedback during gonad development (Figs. 3 and 4) [40,41]. Furthermore, it was observed that the sensitivity of FSH and LH was different in response to the Kiss2-10 peptide. We suspected that the conflicting results of these

fish species may be corrected with the development stage and methods of spawning. In addition, administration of the Kiss2-10 peptide only improved ovary development, consistent with our previous study which identified the gonadal kisspeptin system (Fig. 5) [16]. We suspected that the regulatory function of kisspeptin in LMB depends on gonad development, which mainly plays a regulatory role in the early developmental stage. Thus, the *kiss2/kiss2r* system was identified independently in the pituitary and gonad, and it is possible that there are autocrine/-paracrine effects of the kisspeptin system on the reproduction of largemouth bass.

In summary, this study was the first to identify the distribution of *kiss2r* messenger-expressing cells in the brain of largemouth bass. Furthermore, our results showed that both FSH- and LH-positive cells exhibited the expression of *kiss2r* messengers in the proximal pars distalis of the pituitary. In addition, both in vivo and in vitro experiments showed that Kiss2-10 peptide administration activated gonadotropin subunit gene expression, stimulated both FSH and LH release and promoted ovary development. Our results suggested that the *kiss2/kiss2r* system was independent in the pituitary gland and, as a hypophysiotropic neuropeptide factor, directly modulates the gonadotroph function of largemouth bass.

Author contributions

Shaojun Liu and Wuhui Li conceived and designed the research, Yan Miu, Zexun Zhou, Fangzhou Hu, Ping Wu and Shi Wang performed the experiments and sampling, Zhongyuan Shen, Lei Zeng, Rurong Zhao and Jie Hu analyzed the data, Wuhui Li wrote the manuscript, Min Tao, Chun Zhang, Qinbo Qin revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.repbre.2023.06.003.

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