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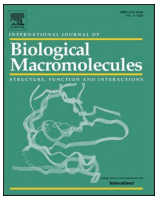

Olfactory receptor OR52N2 for PGE_2 in mediation of guppy courtship behaviors☆

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ABSTRACT

Prostaglandins (PGs) are a type of physiologically active unsaturated fatty acids. As an important sex pheromone, PGs play a vital role in regulating the reproductive behaviors of species by mediating nerve and endocrine responses. In this study, guppy (*Poecilia reticulate*) was used as the model specie to detect the function of PGE_2 in inducing the onset of courtship behaviors. Our results showed that adding PGE_2 into the water environment could activate the courtship behavior of male guppy, indicating that the peripheral olfactory system mediated the PGE_2 function. Thereafter, the open reading frame (ORF) of olfactory receptor *or52n2* was cloned, which was 936 bp in length, coding 311 amino acids. As a typical G protein-coupled receptor, OR52N2 had a conservative seven α-helix transmembrane domains. To confirm the regulatory relationship between OR52N2 and PGE_2, dual-luciferase reporter assay was employed to verify the activation of downstream CREB signaling pathways. Results showed that PGE_2 significantly enhanced CRE promoter activity in *or52n2* ORF transient transfected HEK-293 T cells. Finally, localization of *or52n2* mRNA were observed in ciliated receptor cells of the olfactory epithelium using *in situ* hybridization. Our results provide a novel insight into sex pheromone signaling transduction in reproductive behavior.

1. Introduction

Since the essential function for the successful fertilization, social behaviors including courtship, mating, and aggression during mating process are key parts of reproduction, therefore ensure the species continuation [1]. Although social motivation and behaviors were mediated by the neural circuits in both sexes, their activation is strongly dependent on circulating gonadal hormones [2]. Gonadal hormones play important roles not only in the sexual differentiation, gametogenesis, but also in the sexual behaviors. Circuits gonadal hormones guide neuronal growth [3], cell apoptosis [4], synaptogenesis [5], cytoarchitecture [6], chemoarchitecture [7] and epigenetic modification [8]. Variation in the environmental gonadal hormones "activates" these circuits to further promote the relevant mating behavior [9]. As the largest population vertebrates, teleost rely on pheromone, released into the water environment during ovulation or spermiation, to communicate social information, then trigger a series of specific mating behaviors such as chasing and touching. These behaviors do not need to be learned and can occur after the gonads mature [10].

Mating behavior of teleost could be regulated by both endocrine and nervous system, in which a variety of hormone are involved [11]. Typically, there are three types of hormones. The first category, called "potentiator", such as gonadotropin-releasing hormone (GnRH), is not necessary for mating behavior, but can enhance the effect [12]. The second one does not activate mating behavior, but is dispensable for the behavior [12]. Sex steroid hormones, like estrogens and androgens, belong to this group. The third can activate sexual behavior directly and quickly [12]. Especially prostaglandins (PGs), a series of molecules synthesized from arachidonic acid (AA) by the cyclooxygenase (COXs), can act as physiological triggers to directly induce the onset of mating behaviors from invertebrate [13] to mammals [14]. In oviparous teleost, male and female release mature eggs and sperm respectively at the same time when mating behavior happens. In goldfish (*Carassius auratus*), ovulation in female accompanied with a peak in $PGF_{2\alpha}$ releases which

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further converted to 15 K-PGF$_{2\alpha}$ then activate olfactory sensory neurons in males to induce courtship behavior [15]. Similar behavioral responses triggered by PGs were also found in Lake trout (*Salvelinus namaycush*) [16] and Peacock blenny (*Salaria pavo*) [17]. In African cichlid (*Astatotilapia burtoni*), the mating behaviors were blocked in the global knockout of receptor of PGF$_{2\alpha}$ animals [18]. On the other hand, teleost can recognize amino acids, steroids, prostaglandins, or bile acids in the surrounding water environment through olfactory receptors(ORs) located in the olfactory epithelium, then the information is transmitted to the central nervous system through olfactory signaling pathways, which in turn complete olfaction [19–21]. In zebrafish (*Danio rerio*), the pheromone prostaglandin F$_{2\alpha}$ was confirmed to specifically activate two olfactory receptors to affect fish reproduction [22].

In oviparous teleost, PGs can activate mating process and female ovulation behavior to complete *in vitro* fertilization. But in ovoviviparous teleost, the reproductive strategy of asynchronous process of mating and fertilization exhibits intrafollicular fertilization and gestation. In a previous study on ovoviviparous teleost black rockfish (*Sebastes schlegelii*), COX1–2 was showed significantly elevated when mating behaviors start [23]. Guppy (*Poecilia reticulate*), which belongs to the *Poecilia* genus in the Poecillidae family, has an ovoviviparous pattern and sperm storage *in vivo*. In females, oocytes are fertilized inside the follicular layers without ovulation and embryos develop within the follicular layers [24]. Previous studies have revealed the reproductive strategy affected by the visual system [25], ecological position [26], neural process [27]. However, less is known about the olfactory receptor system mediating pheromone activating mating behaviors. In the present study, aimed to clarify the response of olfactory receptor OR52N2 to the prostaglandin, we analyzed the expression and location of olfactory receptor *or52n2* and tested the downstream signaling of *or52n2* after PGE$_2$ treatment. These findings provide novel results supporting the function of sex pheromone prostaglandin E$_2$ and its cognate olfactory receptor in fish reproductive behavior.

2. Materials and methods

2.1. Animals and ethics statement

The guppies were obtained from Nanshan Fisheries Market (Qingdao, China). All the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Ocean University of China. To minimize suffering of the animals, experimental fish were anesthetized with 3-aminobenzoate methanesulfonic acid (MS-222, 100 ng/mL) before sacrificing and handling. The field studies did not involve endangered or protected species.

2.2. PGE$_2$ induces courtship behavior in guppy

After acclimation, the six guppies were separated into two groups and each group contained a female and a male which were randomly allocated into 3 square aquaria as triplicates. The experimental group was added with 30 nM PGE$_2$ and the control group was treated with the same volume of carrier (absolute ethanol). The behavior changes of male and female guppies were observed and analyzed within 15 min.

2.3. Total RNA extraction and reverse transcription

Three male guppies were selected. The guppies were so small that the olfactory sacs were hard to obtain. Their heads were cut from the snout to the posterior edge of both eyes, total RNA was extracted using TRIzol reagent (Invitrogen, America) according to the reagent instructions. RNA quantity and purity were assessed by a Biodropsis BD-1000 nucleic acid analyzer (OSTC, China) and electrophoresis using a 1 % agarose gel. cDNA was prepared using the Prime ScriptTM RT real Time kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) according to the manufacturer's instructions.

2.4. Molecular cloning and sequence analysis

According to the genome (LR880657.1) and RNA-seq data (XM_016661575.1), the open reading frame (ORF) of *or52n2* in guppy was predicted, and the cloning primers were designed by Primer Premier 5 software. All the primers used in the present study are listed in Table 1. The 2 × Phanta Max Master Mix (Dye Plus) (Vazyme, China) was used for cloning and olfactory sac cDNA was used as the template. The PCR products were purified using a TIANgel Midi purification kit (TIANGEN Beijing, China), subcloned into the PEASY-T1 vector (TransGen Biotech, China), and sequenced.

The amino acid sequence of OR52N2 was predicted by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder). Clustal X software was used to perform multiple alignments of the amino acid sequences of several species. The tertiary structure was accessed using SWISS-Model (https://swissmodel.expasy.org/). The protein molecular weight and theoretical isoelectric point (pI) were configured by ProtParam (http://web.expasy.org/protparam). A phylogenetic tree was constructed with the neighbor-joining method by MEGA-X. The values on the trees indicate the credibility of each branch, representing bootstrap scores of 1000 iterations.

2.5. Cell culture and transfection

The *or52n2* cDNA of guppy was subcloned into the pcDNA3.1 expression vector (Invitrogen, USA). Human embryonic kidney cell lines (HEK-293 T) were resuspended in complete medium DMEM (SparkJade, China) containing 10 % fetal bovine serum (FBS, BioInd, Israel) and were placed in a CO_2 incubator (37 °C) for 3–4 passages. Twenty hours before transfection, 1 × 10^5 cells/well were seeded into 24-well tissue-culture plates. 500 ng CRE-luciferase reporter plasmid, 300 ng pcDNA3.1-*or52n2* of guppy and 50 ng pRL-TK containing the Renilla luciferase reporter gene were co-transfected into the 293 T cells in 50 μL serum-free medium using lipofectamine 3000 reagent (Invitrogen, USA). Six hours after transfection, cells were incubated with various (from 10^{-7}-10^{-5} M) concentrations of PGE$_2$ for a further 48 h.

The cell lysate supernatant then was aspirated by centrifugation at 8,000 rpm (4 °C) and the activity value was determined using a Dual-Lucy Assay Kit (Promega, Madison, USA). The ratio of firefly luciferase to Renilla sea cucumber luciferase was the relative luciferase activity value.

2.6. Haematoxylin-eosin (H&E) staining and in situ hybridization (ISH)

The heads of male guppy were collected and fixed in buffered 4 % paraformaldehyde (in PBS, pH 7.4) for 18 h. After that, the heads were decalcified by 0.25 M EDTA buffer (pH 7.4) for 24 h and then the tissues were dehydrated in a graded series of ethanol, embedded in paraffin and sectioned at a thickness of 5–7 μm using microtome (Leica, Wetzler, Germany).

The sections for H&E staining were dewaxed, rehydrated and stained

Table 1
primers sequences used for ORF cloning and ISH.

Primers	Sequence (5′-3′)
Primers for ORFs clone	
ORF-F	CTTGGTACCGAGCTCGGATCCATGATGGA ACCATTGAAGGAAAA
ORF-R	GGTTTAAACGGGCCCTCTAGACTACCGAT AAGAAAACAGCACTTTG
Primers for ISH	
ISH-F	CGCATTTAGGTGACACTATAGAAGCGTGAT GGAACCATTGAAGGG
ISH-R	CCGTAATACGACTCACTATAGGGAGACAAT CCAACGCCATCCAGACC

with haematoxylin-eosin. Then sections were dehydrated, coverslipped and observed by an Olympus bright field light microscope (Olympus, Tokyo, Japan).

The section for ISH was then dewaxed, rehydrated by successively immersing in the following RNase-free solutions: 0.2 M HCl for 10 min and 10 μg/mL proteinase K (in PBS) for 2 min. The tissue sections were prehybridized at 55 °C for 1 h, and then hybridized with DIG-labeled riboprobes diluted in hybridization buffer at 55 °C overnight in a wet box. After overnight hybridization, the sections were washed in a pre-heated series of SSC (55 °C, 15–30 min for each step) and maleic acid buffer, followed by blocking reagent (Roche Diagnostics, Germany). According to the reagent, DIG was detected with an alkaline phosphatase-conjugated anti-DIG antibody (1:400 dilution), and chromogenic development was conducted with an NBT/BCIP stock solution (Roche Diagnostics).

2.7. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Data analysis was performed by one-way ANOVA followed by Tukey-HSD multiple range tests and differences were considered significant at $P < 0.05$. All the statistical processes were performed using SPSS 20.0 software (SPSS, USA).

3. Results

3.1. PGE_2 induces courtship behavior in guppy

To assess behavioral change, courtship behaviors were categorized as swim, chase and touch. The fish showed a robust attractive response to prostaglandins. When PGE_2 was added, the male chased and touched female more frequently. (Supplementary Video). We recorded the preference of individual fish for every second to quantify the change of behavior. Although the number of chasing and touching was not remarkably changed, the swimming behavior of males to females were significantly increased, indicating that PGE_2 successfully initiated the courtship behavior of male guppies (Fig. 1).

3.2. Gene cloning and in silico sequence analysis of *or52n2* in guppy

The cDNA sequence of *or52n2* gene was identified through genomic data (LR880657.1) and RNA-seq data (XM_016661575.1) mining. The ORF of *or52n2* is 936 bp long and encodes a precursor protein with 311 residues (Fig. 2A). *In silico* analysis showed that OR52N2 is a G protein-coupled receptor with 7-transmembrane domains (Fig. 2B). The physicochemical properties were determined as follows: the molecular weight of OR52N2 protein was 34.77 kDa and the pI was 8.93.

The alignments of guppy OR52N2 amino acid sequences showed that 7- transmembrane structural domains were highly compared with other vertebrate proximal olfactory receptors (Fig. 3A). Phylogenetic analyses also revealed the evolutionary conservation of olfactory receptors

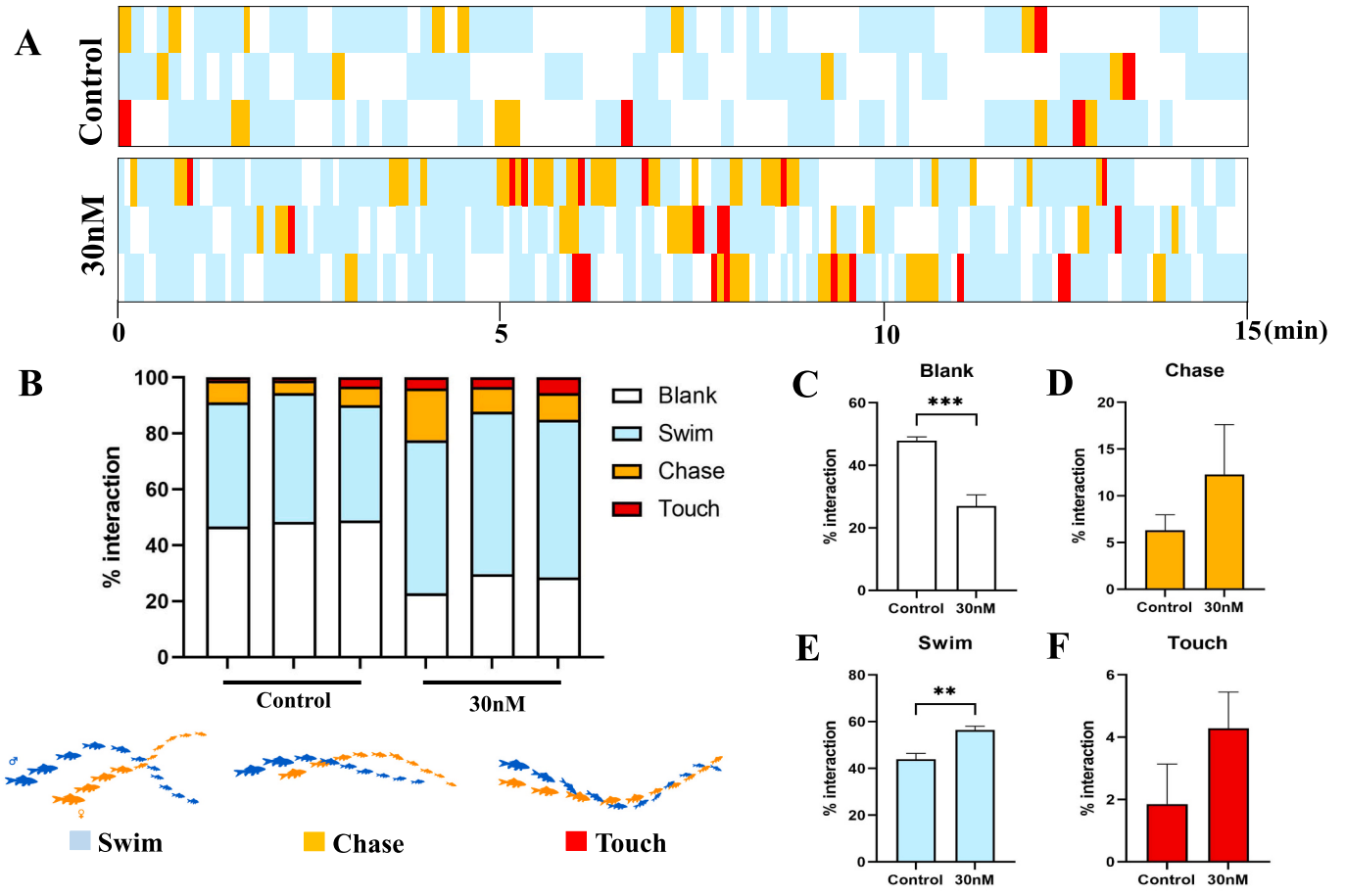

Fig. 1. (A)Mating behavior heatmap of three male and three female guppies under 30 nM of PGE_2 in 15 min. (B)The statistics of male and female behavioral interactions, including chasing, swimming and touching. (C-F) Comparison of different behaviors between control and experimental group. The data are shown as the mean ± S.E.M. ($n = 3$). Data analyzed by one-way ANOVA followed by Tukey analysis. Two and three asterisks indicate significant difference ($P < 0.01$ and 0.001, respectively). Different color box indicated different behavior pattern of male and female guppies.

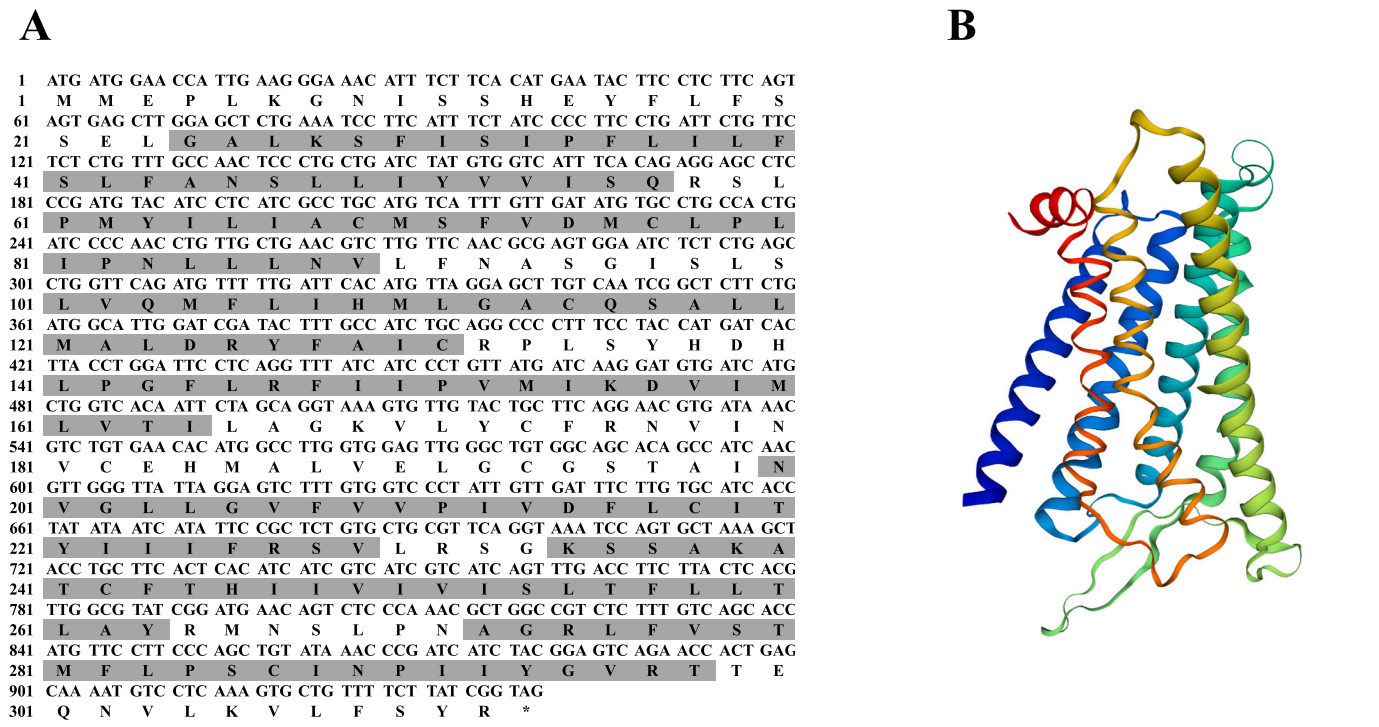

Fig. 2. (A)CDS and deduced amino acid sequences of *or52n2* gene in guppy. (B)Three-dimensional structure prediction of OR52N2 protein was performed, and 7 α-helix transmembrane structures were labeled with different color matching.

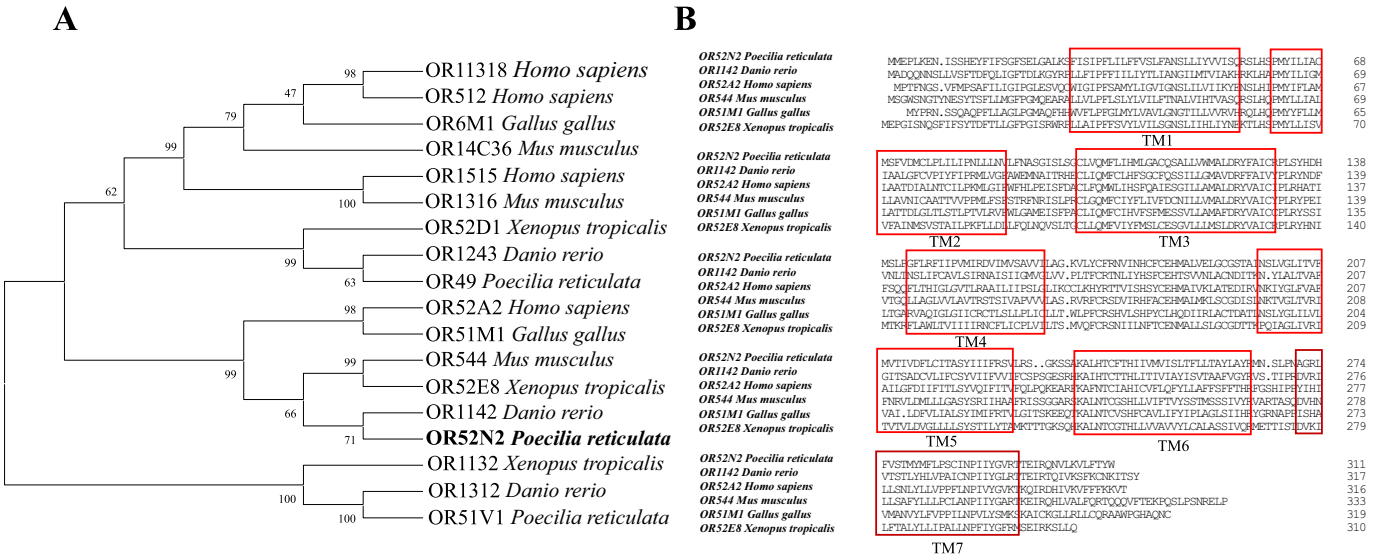

Fig. 3. Phylogenetic tree and multiple sequence alignment of OR52N2. (A)Phylogenetic analysis of ORs in different species. Phylogenetic tree was constructed by MEGA6 software and the neighbor-joining method. The data were resampled with 1000 bootstrap replicates. (B) Multiple comparisons of amino acid sequence of ORs, the red boxes indicated the conversed 7-transmembrane domains. The GeneBank accession numbers of the ORs are as follows: *Homo sapiens* OR52A2 (ALI87744.1), *Homo sapiens* OR11318 (NP_001005194.2), *Homo sapiens* OR1515 (NP_001005326.1), *Homo sapiens* OR512 (NP_666935.1), *Mus musculus* OR1316 (AAP71705.1), *Mus musculus* OR544 (AAI26871.1), *Mus musculus* OR14C36 (XP_025011673.1), *Gallus gallus* OR6M1 (XP_025011542.1), *Gallus gallus* OR51M1 (XP_001008754.1), *Xenopus tropicalis* OR52D1 (XP_031752107.1), *Xenopus tropicalis* OR52E8 (XP_002942417.2), *Xenopus tropicalis* OR1132 (XP_018117821.1), *Danio rerio* OR1243 (ABC43246.1), *Danio rerio* OR1142 (XP_009289741.1), *Danio rerio* OR1312 (NP_001034727.1) , *Poecilia reticulata* OR49 (XP_008402315.1), *Poecilia reticulata* OR51V1 (XP_008423105.1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

among guppy and other vertebrates (Fig. 3B).

3.3. Determination of or52n2-mediated PGE_2 signaling pathway in guppy

The CRE-luciferase assay was performed to evaluate the affinity of PGE_2 on *or52n2*. The ORF of *or52n2* (pcDNA3.1) and pGL3 (CRE-luciferase) and pRL-TK were co-transfected into HEK-293T cell line for further luciferase activity assay. Results showed that, compared with the control group, 10^{-7} M and 10^{-6} M of PGE_2 groups had a similar effect on enhancing CRE promoter activity. However, CRE luciferase activity was significantly ($P < 0.0001$) enhanced under 10^{-5} M PGE_2 compared control group. And the luciferase activity under 10^{-5} M was also

significantly higher than which under 10^{-6} M and 10^{-7} M ($P < 0.001$) (Fig. 4). Furthermore, the CRE-luciferase reporter assay demonstrated a dose-dependent response to PGE_2. In conclusion, PGE_2 activated downstream CREB signaling pathways mediated by *or52n2* and significantly enhanced CRE promoter activity in HEK-293T cells.

3.4. Localization of or52n2 in guppy

As shown in Fig. 5, multiple cellular differentiation of the olfactory nerve and olfactory epithelium were clearly visible in the olfactory sac of guppy, including ciliated receptor cells, ciliated non-sensory cells, supporting cells, and basal cells (Fig. 5B). mRNA localization of *or52n2* was performed in guppy *via* ISH. Positive signals of *or52n2* mRNA were observed in ciliated receptor cells of the olfactory epithelium (Fig. 5C, D).

4. Discussion

Courtship behavior is a significant process, especially the male mate choice [28]. Sexual selection has two major processes: intra-sexual (usually male-male competition) and inter-sexual selection (usually female choice) [29]. When the guppy chooses a mate, males have a directional preference for higher activity levels in females [28]. And females exhibit different preferences towards a series of variably-colored males [30]. PGs, especially the role of PGEs, in ovulation have been well characterized [31,32]. Our results indicated that male guppy chased and touched female guppy several times to complete their courtship behavior under the induction of PGE_2. PGE_2 plays an indispensable role in decidualization, ovulation, implantation, and pregnancy in mammals [33]. In medaka [34] and zebrafish [35], PGE_2 is also

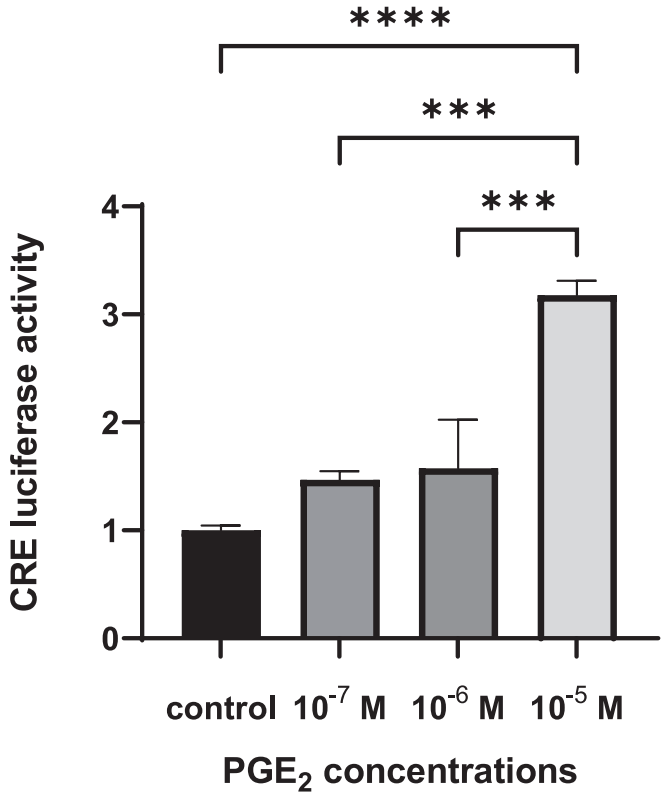

Fig. 4. Activity of CRE in combination with PGE_2 and *or52n2*. The X axis indicates different PGE_2 concentrations (0, 10^{-7} M, 10^{-6} M, 10^{-5} M). The Y axis indicates CRE luciferase activity. The data were shown as the mean ± S.E.M.($n = 3$). Data analyzed by one-way ANOVA followed by Tukey-HSD multiple range tests. Three and four asterisks indicate significant difference ($P < 0.001$ and 0.0001, respectively).

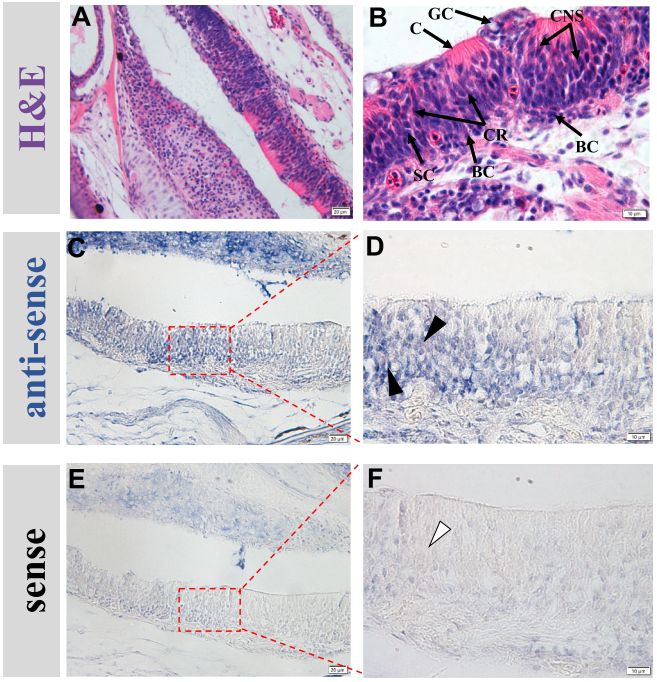

Fig. 5. H&E staining of the olfactory sac in guppy (A, B). Localization of *or52n2* mRNA in the olfactory epithelium of guppy *via in situ* hybridization (C, D). Antisense probe signal of *or52n2* presented in ciliated receptor cells. The arrowhead indicates positive signals in the ciliated receptor cells (C, D). Negative signal with sense probes at same position (E, F). ON: olfactory never; C: cilia; CR: ciliated receptor cell; CNS: ciliated non-sensory cell; GC: goblet cell; SC: supporting cell; BC: basal cell. Scale bars (A, C, E) = 20 μm, Scale bars (B, D, F) = 10 μm.

associated with ovulation. Additionally, another PG, $PGF_{2\alpha}$ was able to activate olfactory receptors in the olfactory sac to trigger male courtship behavior in zebrafish [36]. Accordingly, PGs play important roles in inducing teleost reproductive behavior that can activate prostaglandin receptors in females and olfactory receptors in males to ensure efficient reproductive behavior with reproductive hormones and sex pheromones.

Teleost rely upon olfaction to enable activities such as mating, locating food, and avoiding predators [37]. The olfactory receptors belong to the G protein-coupled 7-transmembrane domain receptors superfamily. OR genes in common carp, goldfish, and cavefish that had 60 %–80 % amino acid identities with zebrafish ORs [36]. The vast majority of vertebrate MORs are single-exon structures with a coding region of approximately 1 kb [38]. The class I MOR may be specialized for detecting water-souble odorants and class II receptors for recognizing volatile odorants [39]. Based on the structure analysis, *or52n2* of guppy may belong to the Main olfactory receptor subfamily and class I receptor gene.

Under the action of signaling pathways, cells can elucidate appropriate physiological responses by monitoring external and internal states [40]. G protein-coupled receptors mediate the cyclic AMP-protein Kinase A (cAMP-PKA) pathway. The effects of PGE_2 in activating cAMP-responsive elements *via or52n2* were detected in HEK-293 T cell lines with luciferase assay. The results indicated that CRE promoter activity mediated by *or52n2* was significantly enhanced with the increase of PGE_2 concentration. We demonstrated a high affinity between PGE_2 and the olfactory receptor *or52n2* in guppy, thus results confirmed the functional relevance of *or52n2* in the peripheral olfactory system regulating courtship behavior.

The olfactory system, composed of olfactory organ, olfactory never, and olfactory bulb, plays a pivotal role in feeding, mating, social behavior, and danger assessment [41]. The olfactory sac, also called

olfactory organ, consists of olfactory sac membrane, rachis, and olfactory lamellae. Guppy is so small that the structure was not observed by H&E staining. Our results indicated that the differentiated cells of the olfactory epithelium were well-organized, and *or52n2* was highly expressed in ciliated receptor cells. In fish, such as catfish [42] and zebrafish [43], the olfactory receptor cells are located in the olfactory epithelium, which is restricted in the olfactory pits [44]. These results indicated a conserved expression profile of the olfactory receptor.

In summary, we have cloned *or52n2* from ovoviviparous guppy and tested its mRNA expression level in olfactory epithelium. Furthermore, we demonstrated that PGE_2 can act as a sex pheromone on the olfactory receptor *or52n2* and subsequently drive courtship behavior in male guppy. Moreover, we also detected the mechanism of the *or52n2* and the mode of action and pathway of PGE_2. Our study provides an important insight into the potential function of olfactory system and sex pheromone in achieving artificial intervention of aquatic animal reproductive behavior.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.124518.

CRediT authorship contribution statement

XQ, HSW, and YL designed the study. JSL and SJY performed the RNA extraction and cDNA preparation. CPZ performed the sequence analysis. YG performed experiment on courtship behaviors. XJ and LKL performed cell culture and transfection. XJW, YJY and SYX performed the haematoxylin-eosin (H&E) staining and *in situ* hybridization (ISH) experiment. XJ and LKL wrote the manuscript. XQ provided manuscript editing and feedback. All authors read and approved the final manuscript.

Declaration of competing interest

There are no conflicts to declare.

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