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Cloning and expression of prostaglandin E_2 receptor subtype 1 (*ep$_1$*) in *Bostrichthys sinensis*

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Abstract Our previous studies suggested that prostaglandin E_2 (PGE_2) is a putative sex pheromone in Chinese black sleeper *Bostrichthys sinensis*, a fish species that inhabits intertidal zones and mates and spawns inside a muddy burrow. We found immunoreactivities of PGE_2 receptor subtypes ($Ep_{1–3}$) expressed in the olfactory sac, but only Ep_1 presented higher density of immunoreactivity in mature fish than that in immature fish in both sexes. To gain a better understanding of the underlying molecular mechanism for the detection of PGE_2 in the olfactory system, we cloned an *ep$_1$* cDNA from the adult olfactory sac. The open-reading frame of the *ep$_1$* consisted of 1,134-bp nucleotides that encoded a 378-amino acid-long protein with a seven-transmembrane domain, typical for the G protein-coupled receptors superfamily. Expression of *ep$_1$* mRNA was observed in all tissues examined, with higher levels obtained in the olfactory sacs and testes. The expression of *ep$_1$* mRNA in the olfactory sacs and gonads was significantly higher in both sexes of mature fish than in those of immature ones. Taken together, our results suggested that Ep_1, which is highly expressed in the olfactory sacs and gonads of mature fish, is important for the control of reproduction and may be involved in PGE_2-initiated spawning behavior in *B. sinensis*.

Keywords *Bostrichthys sinensis* · PGE_2 · Ep_1 · Sex pheromone · Olfactory system

Introduction

Animals demonstrate their reproductive readiness in various ways in order to find mating partners. Many animal species, including fishes, employ sex pheromones during their reproductive seasons in order to attract mating partners (Wyatt 2003). It has become increasingly evident that many fish species release steroids (e.g., *Carassius auratus*), prostaglandins (e.g., *Salmo salar*) or other metabolites (e.g., *Petromyzon marinus*, *Oncorhynchus masou*) during gamete maturation as exogenous signals that trigger mating behavior (Dulka et al. 1987; Sorensen et al. 1988; Moore and Waring 1996; Li et al. 2002; Yambe et al. 2006). Furthermore, electro-olfactogram (EOG)

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recording studies in more than 100 fish species (from the Cypriniformes, Siluriformes, Salmoniformes, Scorpaeniformes and Perciformes) (Sveinsson and Hara 2000) demonstrate that the olfactory system of teleosts is an important seat for processing reproduction-related or pheromone-triggered signals (Biju et al. 2003). However, the molecular mechanisms underlying the detection of sex pheromones in the teleost olfactory system are not well understood.

Signal transduction in the olfactory system begins with the binding of an odorant ligand to a receptor on the olfactory neuron cell surface, initiating a cascade of enzymatic reactions that results in the production of a second messenger and the eventual depolarization of the cell membrane (Breer 1994). The odorant receptors are believed to be members of the superfamily of G protein-coupled receptors (GPCRs) that recognize diverse ligands (Zhao et al. 1998).

The Chinese black sleeper (*Bostrichthys sinensis* Lacepede) belongs to the family Eleotridae and the order Perciformes. This species inhabits intertidal zones. In the mudflats, the fish build 'Y'-shaped muddy burrows of 40–65 cm depth, with one entrance and one exit. During the spawning season, a pair of fish mates and spawns inside a burrow (Hong et al. 2004). This spawning behavior suggests that mature males and females may release sex pheromones to attract each other and to elicit courtship and spawning behavior. In response to prostaglandin E_2 (PGE_2), EOG values are higher in mature fish than in immature ones (Ma et al. 2003). Moreover, PGE_2 concentrations in water samples obtained from spawning pairs are higher than those from non-spawners. Further analyses show that artificial nests with a PGE_2-releasing tube inside attract more males and females than the control and result in the highest percentage of spawning (Hong et al. 2006). All this evidence indicated that PGE_2 might act as a sex pheromone in this species.

To understand the molecular mechanisms of PGE_2 in inducing sexual behavior, we focused on the receptor for PGE_2. PGE_2 exerts its potential by acting on a group of GPCRs. There are four GPCRs that respond to PGE_2, designated as subtypes Ep_1, Ep_2, Ep_3, and Ep_4 (Narumiya et al. 1999). In a previous study, using immunocytochemistry, we found PGE_2 receptors in the complete olfactory system of *B. sinensis* (Lai and Hong 2010). In the olfactory sacs, among $Ep_{1–3}$, only Ep_1 presents a significantly higher density of immunoreactivity in mature fish than that in immature fish in both sexes. Moreover, the density of Ep_1 immunoreactivity in olfactory epithelium is higher (above 7- to 20-fold) than that of $Ep_{2,3}$. These results suggest that Ep_1 may play reproduction-related functions in the olfactory epithelium. To gain a better understanding of the underlying molecular mechanism for the detection of PGE_2 in the olfactory sac, molecular technologies were used in the present study. We first cloned the full-length *ep1* cDNA from the *B. sinensis* olfactory sac and further analyzed its expression pattern in adult fish tissues. Finally, the changes in *ep1* mRNA levels in the olfactory sac were analyzed in different reproductive statuses.

Materials and methods

Animal and tissue sampling

Adult Chinese black sleepers (*B. sinensis*) were collected from the Jiulong River Estuary, Fujian, China, during the spawning (May and June, 2010) and non-spawning seasons (December 2009 and January 2010). Body length and body weight ranges were 162–166 mm and 58.5–75.6 g for females, and 160–177 mm and 51.5–67.5 g for males. The sexual maturity of males was confirmed by applying pressure on the abdomen to check for milt flow. For sexually mature females, eggs could be squeezed out by gentle pressure on the abdomen.

For tissue sampling, the fish were anesthetized with 0.2 % 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St. Louis, MO, US) before being killed. The gonadosomatic index (GSI) was calculated as GSI (%) = gonad weight (g) × 100/total body weight (g).

Cloning and sequence analysis of *ep1* and *β-actin* cDNA

Total RNA was extracted from the adult olfactory sac using Trizol reagent following the manufacturer's instructions (NRC, USA) and reverse-transcribed to cDNA using a RevertAid First Strand cDNA synthesis Kit (Fermentas, Canada). To obtain a partial *ep1* and *β-actin* cDNA sequence, olfactory cDNAs were used as a template in PCR with a primer set (Table 1) corresponding to highly conserved amino acid sequences found in known Ep_1 and β-actin. The following thermal cycling parameters were used:

Table 1 Primer sequences used in this study

Primers	Primer set (5′ → 3′)
First round of RT-PCR	
β-actin F	AGACCTTCAACACCCCHGCCAT
β-actin R	ACTCCTGCTTGCTRATCCACAT
ep_1 F	GCCVGGSACKTGGTGCTTCAT
ep_1 R	TAMACCCANGGRTCSARDATCTG
ep_1 5′, 3′-RACE	
3′GSP1	CTGCCGTTTGGTGATGCGATA
3′GSP2	AGTGCGGACATCAACCCAAAGA
Outer primer	CATGGCTACATGCTGACAGCCTA
Inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
5′GSP1	CTGCCGTTTGGTGATGCGATA
5′GSP2	AGTGCGGACATCAACCCAAAGA
Real-time PCR	
β-actin F-q	GACAGGTCATCATCATTGGC
β-actin R-q	CAGACAGCACAGTGTTGGCATAC
ep_1 F-q	CAAGACTAAAGAAGACGACCTGC
ep_1 R-q	AGTGCGGACATCAACCCAAAG

94 °C for 3 min (1 cycle); 94 °C for 30 s, 56 °C for *β-actin* and 58 °C for ep_1 for 1 min and 72 °C for 1 min (35 cycles) followed by a final DNA extension at 72 °C for 5 min. All PCR products were then purified from agarose gel and sub-cloned into vector PTZR/T (InsTAclone PCR Cloning Kit, Fermentas, Canada), and then, the plasmid DNA of several positive clones was prepared for DNA sequence analysis.

To obtain full-length ep_1, olfactory sac total RNA was reverse-transcribed to 5′- and 3′-RACE ready cDNA using either a 5′-Full RACE Kit or a 3′-Full RACE Core Set (Takara, Japan), in accordance with the manufacturer's instructions. We isolated the 5′- and 3′-ends of the ep_1 cDNA, using gene-specific primers (Table 1). Based on the partial cDNA sequence obtained, gene-specific primers were designed for further extension by 5′- and 3′-RACE. These initial 5′- and 3′-RACE products were then used for nested PCR amplification with gene-specific nested primers (GSP). The PCR products were sub-cloned and sequenced. All RACE reactions were carried out following the manufacturer's instructions.

Phylogenetic analysis

After obtaining the *B. sinensis* ep_1 cDNA sequence, a BLAST homology search was performed using the deduced amino acid sequences. The alignment of multiple Ep_1 protein sequences from different vertebrate species was performed using the Megalign program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) with the Clustal V (PAM 250) algorithm. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987).

Quantification of ep_1 expression

To assess ep_1 mRNA expression in different tissues of *B. sinensis*, olfactory sac, olfactory bulb, telencephalon, gill, muscle, liver, spleen, intestine, stomach, ovary, seminal vesicle and testis samples were collected from three females or males. In addition, olfactory sac, stomach, ovary and testis tissues were also collected during the spawning and non-spawning seasons, placed into liquid nitrogen and then stored in a −80 °C refrigerator. Total RNA was isolated, treated with DNase I (Fermentas, Canada) and reverse-transcribed to cDNAs as described above. Samples of the cDNAs were diluted 1:10 prior to use as templates for quantitative real-time PCR (qPCR) using a SYBR GREEN 2 × Premix Kit (Takara, Japan) following the manufacturer's instructions. The specificity and efficiency of these primer sets (Table 1) were examined using serial dilutions of

olfactory sac cDNAs. All qPCR were performed in 20 μL reactions, and Ct values were determined. The amplification protocol was 95 °C for 30 s (1 cycle); 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s (40 cycles). A melt curve analysis was performed at the end of the reaction to check the reaction specificity. Relative *ep1* mRNA levels were calculated using the standard curve method (Morrison et al. 1998). The Ct values of *β-actin* and *ep1* in target tissues can be translated into a quantitative result using the constructed standard curves. Subsequently, *ep1* mRNA levels were normalized to the reference gene *β-actin*, which remained stably expressed under the different experimental conditions (Supplemental Figures 1, 2).

Statistical analysis

The data drawn from all the fish tissues belonging to each reproductive phase were pooled and averaged. One-way ANOVA followed by Tukey's post hoc test was used to determine whether significant differences in *ep1* expression levels existed between different tissues, and Student's *t* test was used to determine whether significant differences in *ep1* expression levels existed in respective tissues between different reproductive statuses.

Results

Cloning of the full-length *ep1* cDNA from *B. sinensis*

We obtained a full-length cDNA of *B. sinensis ep1* with 1,502 bp (GenBank Accession No: JF946753) encoding 378 amino acids (Fig. 1). Analysis using the TMHMM program (www.cbs.dtu.dk/services/TMHMM/) revealed that the deduced amino acid sequence had seven transmembrane domains with the N terminus on the outside of the cell and the C terminus on the inside of the cell. Conserved motifs for prostanoid receptors were also found in *B. sinensis* Ep_1, including GTYTY-QYPGTWCF in the second extracellular loop, and MVFFGLCPLLLGCAMAAER and NQILDPWVYIL in the third and seventh transmembrane domain, respectively (Fig. 1). Phylogenetic analysis showed that fish and mammalian Ep_1 were grouped into the two major clades (Fig. 2). *B. sinensis* Ep_1 had a higher homology with Ep_1 in other teleosts (~62.1 %) compared with

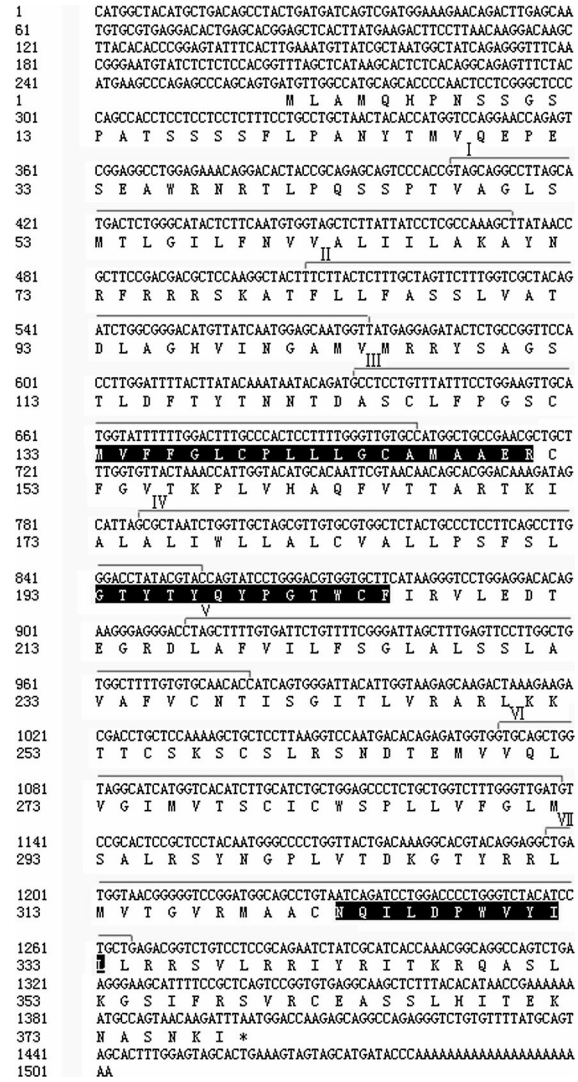

Fig. 1 Full-length cDNA of *ep1* and encoded amino acid sequence cloned from *B. sinensis*. The numbers on the *left* of the sequence show the positions of the first nucleotide and amino acid on each *line*, respectively. Positions of the putative transmembrane domains are indicated by the *upper lines*. Conserved motifs are *shaded*. The GenBank Accession number is JF946753

those in mammals (~41.3 %). The *B. sinensis* Ep_1 shared a high amino acid sequence identity with zebrafish Ep_{1b} (62.1 %), which was comparably higher than those of zebrafish Ep_{1a} and Ep_{1c} (58.3 and 41.3 %).

Expression of *ep1* in various tissues of *B. sinensis*

Expression of *ep1* mRNA was found in all the tissues examined (Fig. 3). Significantly ($P < 0.05$) high

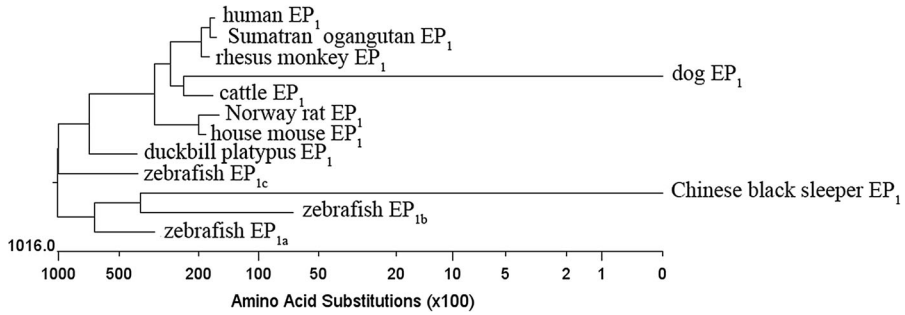

Fig. 2 Phylogenetic analysis of EP_1. Cattle EP_1 accession number: XP_584035.3; Chinese black sleeper EP_1 accession number: JF946753; dog EP_1 accession number: XP_853418.1; duckbill platypus EP_1 accession number: XP_001516507.1; house mouse EP_1 accession number: BAB29498.1; human EP_1 accession number: AAP32302.1; Norway rat EP_1 accession number: NP_037232.1; rhesus monkey EP_1 accession number: NP_001028205.1; Sumatran orangutan EP_1 accession number: XP_002828836.1; zebrafish Ep_{1a} accession number: NP_001159805.1; zebrafish Ep_{1b} accession number: NP_001159802.1; and zebrafish Ep_{1c} accession number: ACX47465.1. The horizontal distances to the branching points are proportional to the number of amino acid substitutions

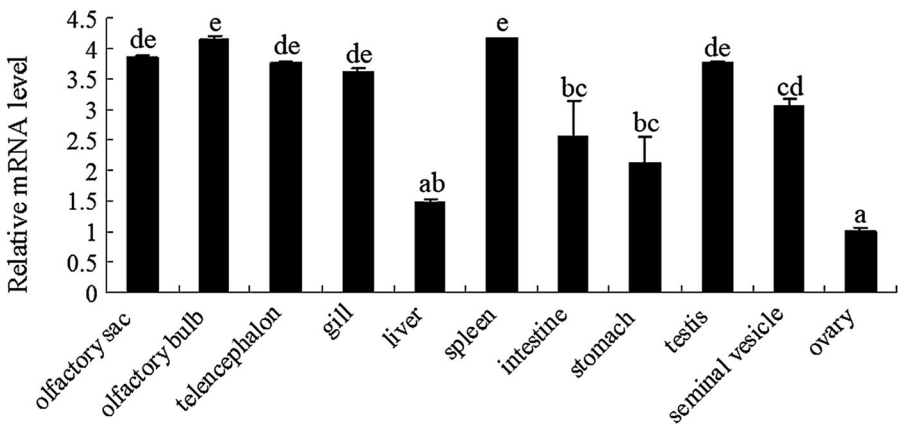

Fig. 3 Relative expression levels of *ep1* mRNA in various tissues of *B. sinensis*. The *ep1* mRNA expression levels were normalized by the expression of *β-actin* mRNA in each tissue. Values represent mean ± SEM ($n = 3$) relative to *ep1* mRNA levels measured in the tissue with the lowest value. *Bars* marked with different letters are significantly different from each other ($P < 0.05$)

expression levels of *ep1* mRNA were found in the olfactory sac, olfactory bulb, spleen and testis, while significantly ($P < 0.05$) low expression levels were observed in the liver and ovary.

High expression of PGE_2 receptor subtype *ep1* mRNA in mature fish

The GSI values of sexually mature males (0.14 ± 0.042 %) and females (7.38 ± 3.0 %) were significantly ($P < 0.05$) higher than those of immature males (0.030 ± 0.0084 %) and females (0.48 ± 0.072 %).

Significantly ($P < 0.05$) higher expression of *ep1* was observed in the olfactory sac in sexually mature fish than in immature ones in both sexes (Fig. 4). Similarly, significantly ($P < 0.05$) higher expression of *ep1* mRNA was also observed in the gonads in mature fish than in immature ones in both sexes. In contrast, no significant differences in *ep1* mRNA expression were found in the stomach tissues of sexually mature fish compared with the immature fish in both sexes (Fig. 4).

Discussion

For many genes, ray-finned fish have two paralogous copies, whereas only one ortholog is present in tetrapods (Wittbrodt et al. 1998). This is related to the teleost-specific genome duplication which occurred after the split of the Acipenseriformes and the Semionotiformes from the lineage leading to teleost fish, but before the divergence of the Osteoglossiformes (Hoegg et al. 2004). Three zebrafish

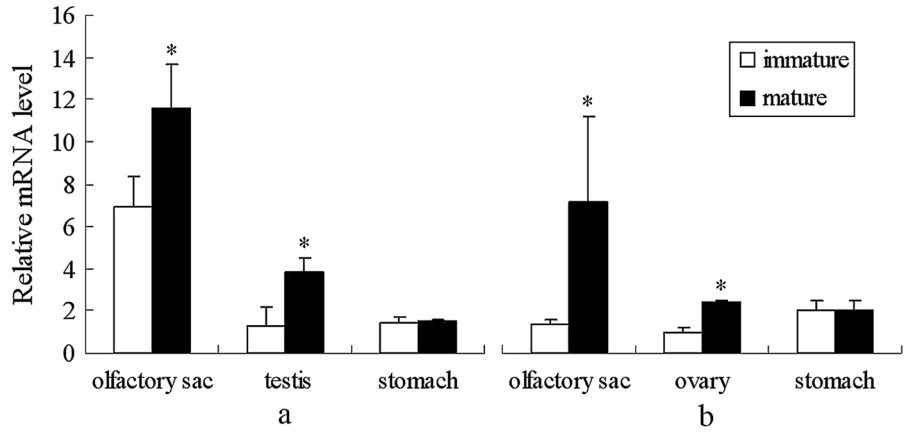

Fig. 4 Relative expression levels of ep_1 mRNA in olfactory sac, ovaries and stomach tissues of male (**a**) and female (**b**) *B. sinensis*. Values represent mean ± SEM ($n = 3$) relative to ep_1 mRNA levels measured in the tissue with the lowest value. *Bars* marked with an * indicate significant difference between mature and immature *B. sinensis* ($P < 0.05$)

prostaglandin E receptor subtype 1 (zep_1) isoforms—zep_1a (GQ911587), zep_1b (GQ911588) and zep_1c (GQ911589)—are identified from the adult ovary (Kwok et al. 2012). However, experimental trials to isolate additional ep_1 cDNAs or in silico approaches to identify related sequences did not provide evidence for the existence of any additional ep_1-like genes or mRNA isoforms from one gene in *B. sinensis*. Thus, it is likely that the *B. sinensis* genome had lost the additional ep_1 gene.

In the mouse (*Mus musculus*), the distribution of ep_1 mRNA is restricted to several organs, such as the kidney, lung and stomach (Watabe et al. 1993). In the brain, ep_1 mRNA is also found in the mouse dorsal root ganglion (Oida et al. 1995) and the paraventricular nucleus of the piglet hypothalamus (Coleman et al. 1994). In the present study, ep_1 mRNA was expressed in all the tissues sampled from adult *B. sinensis*, with a relatively higher expression in the brain tissues. This expression pattern suggests that Ep_1 mediates a vast range of PGE_2 actions in teleost fish, including gastric motor activity and emptying, inhibiting sodium salt absorption in collecting tubules, sensitization of neurons to nociception, inhibition of sleep induction and stress-induced impulsive behavior (Breyer et al. 1998; Matsuoka et al. 2005; Mizuguchi et al. 2010; Moriyama et al. 2005; Yoshida et al. 2000). It is interesting that in the present study, ep_1 mRNA was highly expressed in the spleen. Studies in mammalian models indicated Ep_1 possibly functions as a potent immunomodulator in spleen B lymphocytes (Phipps et al. 1991). Further study in mouse indicated that EPs are sufficient for mediating PGE-induced growth inhibition of susceptible B lineage cells (Fedyk et al. 1996). Besides, the high expression levels of ep_1 mRNA in spleen may suggest spleen plays an important role in control of PGE_2 synthesis in *B. sinensis*. However, our previous study indicated that sex organs serve as major sources of sex pheromones/PGE_2 in *B. sinensis* (Hong et al. 2006). Therefore, the major function of PGE_2 synthesis in spleen may be related to local homeostasis (Smith and Langenbach 2001; North et al. 2007), but not released into water as sex pheromones. The Ep_1-mediated PGE_2 function on spleen of *B. sinensis* needs to be further investigated.

A recent study in mice also demonstrates that the four PGE_2 receptor subtypes (EP_1, EP_2, EP_3 and EP_4) are expressed in the olfactory epithelium (Fukuiri et al. 2013). EP_4 is the most abundantly expressed, the expression of EP_2 is moderate, that of EP_3 is mild and EP_1 is hardly detectable. However, so far, we have not been successful in cloning $ep_{2–4}$ in the olfactory sac of mature *B. sinensis*. Also, a transcriptome analysis did not detect $ep_{2–4}$ in the olfactory sac in immature *B. sinensis* (data unpublished). It is worth noting that our previous study indicates that the density of Ep_1 immunoreactivity in olfactory epithelium is significantly higher (above 7–20 fold) than that of $Ep_{2,3}$ in both sexes regardless of the reproductive stages (Lai and Hong 2010). Further study is necessary to clone $ep_{2–4}$ using genomic DNA as template in the spawning season or using cDNA template from an *eps* highly expressed organ, i.e., the kidney.

Our present study revealed that the PGE_2 receptor subtype ep_1 mRNA was present in the olfactory sac and gonad of *B. sinensis* and that its expression was significantly higher in mature *B. sinensis* than in immature *B. sinensis*. These results coincide with our

previous immunocytochemistry and electrophysiology studies (Lai and Hong 2010; Ma et al. 2003), which demonstrate that the number of immunoreactive olfactory receptor neurons and the EOG of the olfactory organ are related to reproductive status in *B. sinensis*. Variations with reproductive status in response to putative sex pheromones are found widely in teleosts (Irvine and Sorensen 1993; Moore and Waring 1995; Belanger et al. 2004). The levels of olfactory sensitivities may be modulated by changes in the number of receptors and/or the sensitivities of the receptors (Creese and Sibley 1981; Habibi et al. 1989). Our results suggest that olfactory sensitivities in response to sex pheromones are related to the number of receptors in *B. sinensis*, which might further explain the season-related variation of olfactory sensitivities in response to sex pheromones in teleosts (Moore and Waring 1996).

Conclusions

In the present study, we cloned a full-length cDNA coding an ep_1 from the olfactory sac of *B. sinensis*. The higher expression of ep_1 mRNA in the olfactory sac in mature males and females suggested that Ep_1 might be a mediator for PGE_2 to act as a sex pheromone. Moreover, our results suggest that the increased olfactory sensitivity to PGE_2 might be related to the number of the Ep_1 in olfactory sac of *B. sinensis*. Further studies are needed to investigate the Ep_1-mediated signal transduction pathway in the olfactory sac of *B. sinensis*.

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