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Cloning and olfactory expression of progestin receptors in the Chinese black sleeper *Bostrichthys sinensis*

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ABSTRACT

Our previous studies suggested that 17α,20β-dihydroxy-4-pregnen-3-one (DHP), an oocyte maturation inducing progestin, also acts as a sex pheromone in Chinese black sleeper *Bostrichthys sinensis*, a fish species that inhabits intertidal zones and mates and spawns inside a muddy burrow. The electro-olfactogram response to DHP increased during the breeding season. In the present study, we cloned the cDNAs of the nine progestin receptors (*pgr*, *paqr5*, *6*, *7*(*a*, *b*), *8*, *9*, *pgrmc1*, *2*) from *B. sinensis*, analyzed their tissue distribution, and determined the expression in the olfactory rosette during the reproductive cycle in female and male fish. The deduced amino acid sequences of the nine progestin receptors share high sequence identities with those of other fish species and relatively lower homology with their mammalian counterparts, and phylogenetic analyses classified the nine *B. sinensis* progestin receptors into their respective progestin receptor groups. Tissue distribution of *B. sinensis* progestin receptors showed differential expression patterns, but all these nine genes were expressed in the olfactory rosette. Interestingly, *paqr*5 mRNA was found in the intermediate and basal parts of the olfactory epithelium but not in the central core using *in situ* hybridization, and its expression level was the highest in the olfactory rosette among the tissues examined. These results suggested Paqr5 may have an important role for transmitting progestin signaling in the olfactory system. The expression levels of *paqr7a* and *paqr7b*, *pgr* and *pgrmc2* mRNA peaked around the mid meiotic stage, and that of *paqr8* peaked at late meiotic stage in the olfactory rosette in males, while the olfactory expression of *paqr5* decreased gradually as spermatogenesis progressed. In contrast, the expression of the progestin receptors did not change significantly during the development of the ovary in the olfactory rosette in females, except that of *pgr*. Interestingly, the changes of *paqr8* expression in the olfactory rosette in males mirrored the changes of plasma DHP levels in females during the reproductive cycle, suggesting the Paqr8 may also be important for deciphering progestin signaling released by female. To our knowledge, this is the first time to demonstrate the presence of all known progestin receptors in a teleost olfactory rosette, and to show different expressions between the males and females during the reproductive cycle. This study provides the first evidence on changes of all purported progestin receptors during a reproductive cycle in teleost olfactory rosette, and suggests that distinct olfactory sensitivities to DHP may be due to the changes and compositions of each progestin receptor in *B. sinensis*.

1. Introduction

Olfactory system plays an important role for achieving reproductive success in teleost. Many fish species employ sex pheromones for locating suitable partners, and evoking complex behavioral and endocrine responses, which also improve synchronization of gametogenesis, spawning, fertility and paternity (Kobayashi et al., 1986; Dulka et al., 1987; Burnard et al., 2008; Huertas et al., 2014). Teleost pheromones are generally categorized into two types: primer pheromones evoke changes in the endocrine or physiological state of conspecifics, and releaser pheromones induce rapid behavioral responses, but several pheromones exert both primer and releaser effects (Sorensen et al., 1989; Stacey and Sorensen, 2005; Kawai et al., 2014).

17α,20β-Dihydroxy-4-pregnen-3-one (DHP), a maturation inducing progestin in many teleost, has also been suggested to be one of the pheromones (Dulka et al., 1987; Stacey and Sorensen, 2005; Kawai et al., 2014). The presence of circulated DHP was first reported by Idler and co-workers (1960) in female sockeye salmon *Oncorhynchus nerka*, and later was demonstrated

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to be a potent inducer for final oocyte maturation in female rainbow trout *Oncorhynchus mykiss* (Fostier et al., 1973). In addition to DHP's important role in germ cell maturation in teleost (Tubbs and Thomas, 2009), DHP is also involved in chemical communication between conspecifics (Scott et al., 2010). The role of DHP as a pheromone is probably best understood in the goldfish *Carassius auratus*. Exposure to DHP, which is released by pre-ovulatory female, activates a population of neurones in ventral pre-optic area (POA) that plays important roles in reproduction, and triggers immediate increases in *cGnRH* mRNA level in the telencephalon, luteinizing hormone (LH) in the plasma and milt volume (Kobayashi et al., 2002; Lado et al., 2013; Kawai et al., 2014). Moreover, electro-olfactogram (EOG) recording studies have shown that olfactory response thresholds to pheromones including DHP vary with reproductive stages of the fish and it is likely that this sensitivity of the olfactory epithelium is controlled by the endocrine system (Irvine and Sorensen, 1993; Cardwell et al., 1995; Belanger et al., 2007). However, the molecular mechanisms for the detection of DHP, and different sensitivity to DHP during the reproductive cycle are not well understood.

Three types of progestin receptors have been identified so far, i.e. the nuclear progesterone receptor (PGR), membrane progestin receptors (mPRs) and progesterone receptor membrane components (PGRMCs). The PGR, a ligand-activated transcription factor in the steroid hormone receptor super-family, has been well studied during the past 45 years (Rondell, 1974; Graham and Clarke, 1997; Hasegawa et al., 2005). In the majority of vertebrates, a single *pgr* gene has been described, which encodes two Pgr proteins (Pgr-A and Pgr-B) from a single locus varying only N-terminal length. Two distinct *pgr* genes encoding two Pgr proteins that differ considerably in the amino acid sequences have also been found in Japanese eel *Anguilla japonica* (Todo et al., 2000; Ikeuchi et al., 2002) and *Xenopus laevis* (Bayaa et al., 2000; Tian et al., 2000). It is well-known that Pgr controls various physiological processes including ovulation, breast development, pregnancy establishment and maintenance via the both genomic and non-genomic signaling pathway in mammals (Richards, 2005; Baldi et al., 2009; Akison and Robker, 2012; Zhu et al., 2015). A recent study revealed that Pgr immunoreactivity is present in the olfactory epithelium in both sexes of the trout *Salmo trutta fario*, suggesting it may be involved in sex pheromone detection (Varricchio et al., 2010). The mPRs belong to progestin and adipoQ receptor family (PAQR) (Zhu et al., 2003; Tang et al., 2005; Thomas et al., 2007); consist of five subtypes: mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6), and mPRε (PAQR9) in all vertebrates. Paqr7 (mPRα) is the most extensively characterized progestin membrane receptor and it is involved in induction of oocyte meiotic maturation (Zhu et al., 2003; Tokumoto et al., 2006; Tubbs et al., 2010; Hanna and Zhu, 2011), sperm hypermotility (Tubbs and Thomas, 2009; Tubbs et al., 2011), inhibition of apoptosis in teleost granulosa cells and in human breast cancer cells (Dressing et al., 2010, 2012), and inhibition of GnRH release from rodent GnRH neurons (Sleiter et al., 2009). Paqr8 has also been suggested to mediate progesterone signaling in inducing oocyte maturation in *Xenopus* (Josefsberg Ben-Yehoshua et al., 2007), and in teleost (Thomas et al., 2004; Tokumoto et al., 2012). Paqr5 is found on the apical membrane of ciliated epithelial cells in fallopian tubes, suggesting it may have a role in regulating ciliary activity and gamete transport (Nutu et al., 2007, 2009). Neuroprotective actions of allopregnanolone and progesterone may be mediated through Paqr6 (Pang et al., 2013). Recently, *paqr7* and *paqr5* mRNAs were found in the olfactory epithelium of Atlantic croaker *Micropogonias undulatus* (Tubbs et al., 2010) and goldfish respectively (Kolmakov et al., 2008), suggesting they could potentially mediate the pheromonal signaling in teleosts. The PGRMCs contain an N-terminal transmembrane domain and a putative cytoplasmic cytochrome b5 domain ligand-binding motif. The Pgrmc1 was first purified from porcine liver microsomal membrane fractions (Meyer et al., 1996) and shown to cofractionate with the endoplasmic reticulum in liver extracts (Nolte et al., 2000). Pgrmc1 may be involved in a wide diversity of functions including axonal guidance, steroid synthesis and metabolism, cholesterol regulation and endocystosis, and epidermal growth factor receptor functions (Cahill, 2007; Rohe et al., 2009; Thomas et al., 2014). However, considerably less information is available on Pgrmc2 expression and function. Recently, Albrecht et al. (2012) demonstrated in SKOV-3 cancer cells that Pgrmc2 may function to inhibit cell migration. Currently, the information on the presence and expression changes of these progestin receptors in olfactory system are very limited, the current study is intended to address this question.

The Chinese black sleeper (*Bostrichthys sinensis*) belongs to the family Eleotridae, suborder Gobioidei. This species is a burrowing animal and inhabits intertidal zones (Wang et al., 2011). It is a seasonal breeding fish. Females and males live in individual burrows during the nonspawning season; then male fish will form individual pair with female, and spawn inside the same burrow during the spawning season (Hong et al., 2006). This spawning behavior and burrow-living habit suggest that mature males and females may employ sex pheromone to synchronize gametogenesis, spawning and fertility inside the burrow, since visual communication seems less efficient under dark condition. Our previous studies showed both mature male and female *B. sinensis* display greater EOG response to DHP, compared to immature fish (Ma et al., 2003). Moreover, artificial nests with a DHP-releasing tube inside attract more males and females and result in higher percentage of spawning than the control (Hong et al., 2006). Exposure to environmental DHP significantly upregulated the *lhβ* mRNA level in the pituitary of male *B. sinensis* during the breeding season but not the non-breeding season (unpublish data). These results suggest that DHP is a putative sex pheromone in *B. sinensis* and the olfactory sensitivity to DHP depends on reproductive status.

To better understand the reproductive roles of progestin receptors in the olfactory system, we first cloned the cDNAs of the nine progestin receptors (*paqr5*, *6*, *7* (*a*, *b*), *8*, *9*, *pgr*, *pgrmc*1 and 2) from *B. sinensis*, then analyzed their tissue distribution and the expression changes in the olfactory rosette during the reproductive cycle in the female and male fish.

2. Material and methods

2.1. Experimental fish

Adult Chinese black sleeper *B. sinensis* were collected from Dadeng island, Fujian, China between December 2013 and June 2014. Body length and body weight were measured (150–169 mm and 72–108 g). Gonadosomatic index (GSI) was calculated as GSI (%) = [gonad weight (g)/total body weight (g)]*100%. All experiment protocols were reviewed and approved by the Institute Animal Care and Use Committee of Xiamen University.

2.2. Cloning of progestin receptors

Olfactory rosette tissue was used for cloning the *paqr5-8*, *pgrmc1* and *pgrmc2*, testis tissue for cloning the *pgr* and brain tissue for cloning the *paqr9* cDNA. Total RNA was extracted using the RNAzol reagent (Molecular Research Center Inc. (MRC), Cincinnati, OH, USA) and reverse transcribed into first strand cDNA using SMART RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. A similar strategy was carried out for obtaining the full cDNA sequences of all nine progestin receptors. For instance, in order to obtain a partial sequence of *paqr5*, we used

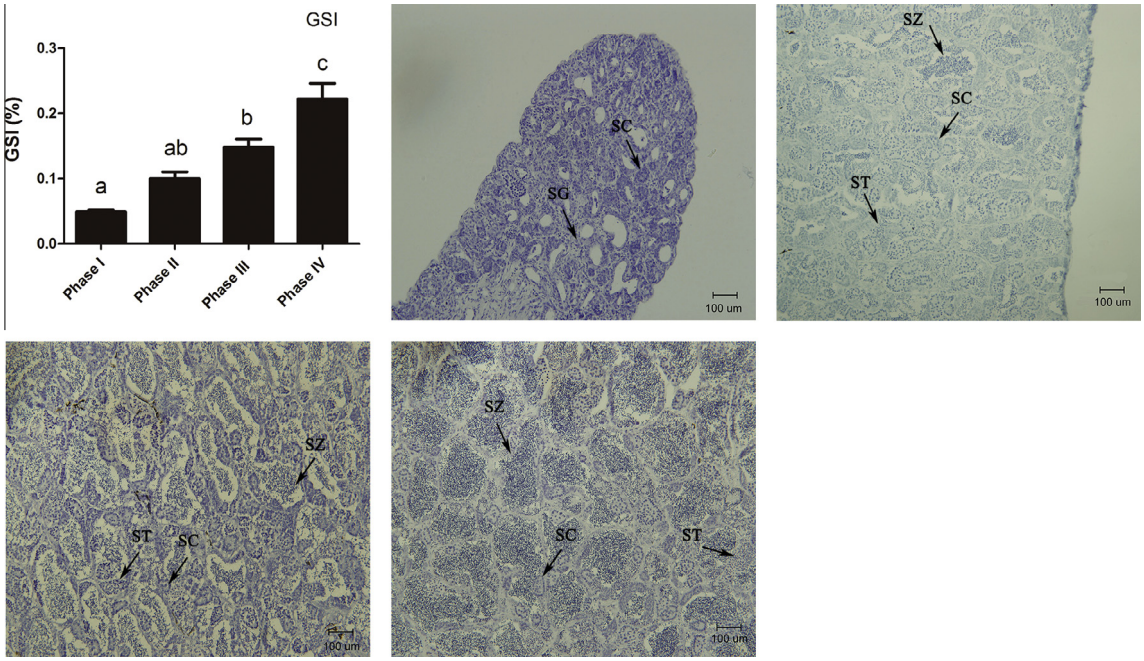

Fig. 1. Gonadosomatic index (GSI) and paraffin sections from male *B. sinensis* showing different phases of testis development. GSI value (A). Data are expressed as the mean ± SEM (n = 6). Bars marked with different letters are significantly different from each other ($p < 0.05$). Phase I, Early meiosis (B), Phase II, Mid meiosis (C), Phase III, Late meiosis (D), Phase IV, Maturation (E). SC, spermatocyte; SG, spermatogonia; ST, spermatids; SZ, spermatozoa.

Table 1
Classification of male maturation state in *B. sinensis* (adapted from Saraiva et al., 2015).

Phase	Classification	Microscopic appearance
I	Early meiosis	Spermatocytes are observed in testis
II	Mid meiosis	Spermatids are observed in testis
III	Late meiosis	All types of germ cells are observed
IV	Maturation	Numbers of spermatogonia and spermatocytes are declining, and the lobule lumen is filled with spermatozoa

degenerated primers designed based on the highly conserved amino acid sequences of known *paqr5* in vertebrates. The PCR amplification was carried out in 20 μl volume under the following cycling conditions: 94 °C for 3 min (1 cycle); 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min (35 cycles) followed by a final extension step at 72 °C for 10 min. All PCR products were purified from agarose gel and sub-cloned into vector pmd19-t (TAKARA, Japan), and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI, USA). The plasmid DNA of several positive clones was prepared for DNA sequencing (Invitrogen Ltd, Guangzhou, China). Based on the partial cDNA sequence obtained, gene-specific primers were designed for further extension by 5′- and 3′-RACE. The first PCR amplification for 5′ or 3′ RACE was performed using a universal primer in the kit and a gene specific primer. If no specific band was obtained, these initial 5′ or 3′ RACE products were diluted and used for nested PCR amplifications with gene-specific nested primers, in combination with a nested universal primer. All RACE reactions were carried out following the manufacturer's instructions. RACE products were sub-cloned and sequenced as described above.

2.3. Phylogenetic analysis

Since membrane progesterone receptors belong to PAQR family, Pgr belongs to nuclear receptors subfamily and Pgrmc1 and 2 belong to membrane associated progesterone receptor (MAPR) family, three phylogenetic analyses were performed separately using a similar strategy. For example, after obtaining the cDNAs of the *B. sinensis*'s five membrane progestin receptors, a BLAST homology search was performed using deduced amino acid sequences. The alignment of known membrane progesterone receptors was performed using MEGA 6.0 program and the Clustal W method. Then a phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 trials for each position and rooted by zebrafish (*Danio rerio*) Paqr10.

2.4. Tissue specific expression of the progestin receptors

During the spawning season in June, four mature male and female fish were anesthetized in 0.01% MS222 (Sigma–Aldrich) buffered solution with an equal amount of sodium bicarbonate and decapitated humanely. The brains were trimmed to collect the olfactory bulb, telencephalon, diencephalon, mesencephalon, cerebellum and medulla oblongata separately. The olfactory rosette, gill, heart, intestine, liver, spleen, skin, muscle, gonad were collected and immediately dipped into liquid nitrogen and stored at −80 °C. Total RNA was extracted from the tissue samples using the RNAzol method (MRC). The same amount of total RNA (1 μg) was used for synthesis of the first strand cDNAs using the RevertAid first strand cDNA synthesis kit (Fermantas). Real-time qPCR was performed as described in the Section 2.7.

2.5. Expression changes of progestin receptors in the olfactory rosette during the reproductive cycle

From December 2013 to June 2014, six male and six female adult fish were anesthetized and humanely decapitated each month. The olfactory rosette was collected and immediately dipped into liquid nitrogen and stored at −80 °C. Total RNA extraction and cDNA synthesis for the olfactory rosette were conducted as described in the Section 2.4. Real-time qPCR was performed as described in the Section 2.6. The testes were removed and fixed in Bouin's reagent overnight for conventional histology

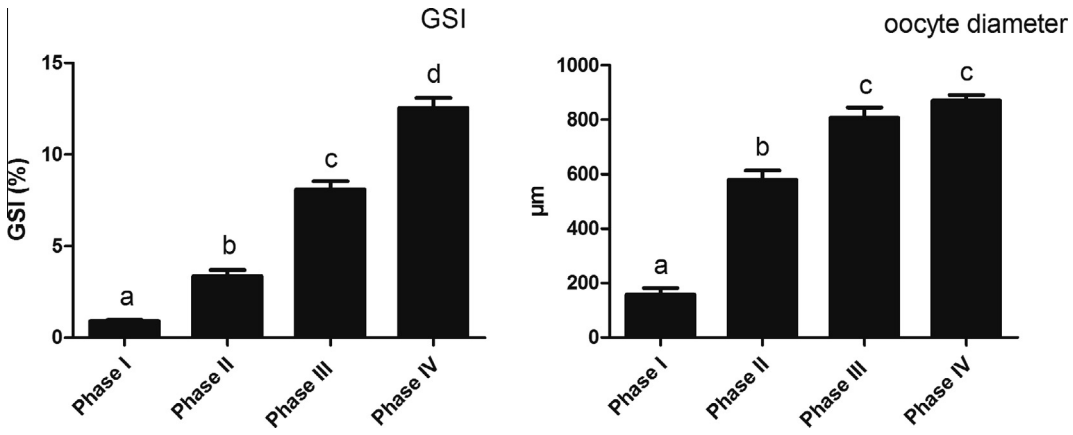

Fig. 2. Gonadosomatic index (GSI) and oocyte diameter of female *B. sinensis*. GSI value (A), oocyte diameter (at least five oocyte were selected from one female fish to determine the mean value) (B). Data are expressed as the mean ± SEM (n = 6). Bars marked with different letters are significantly different from each other ($p < 0.05$).

Table 2
Classification of female maturation state in *B. sinensis* (adapted from Hong et al. 2009).

Phase	Classification	Oocyte diameter (μm)	GSI value (%)	Macroscopic description
I	Immature	<200	<2	Ovary semitransparent, few immature oocytes visible
II	Maturing	200–600	2–4	Ovary pale yellow, opaque oocytes visible and inseparable
III	Nearly ripe	600–800	4–8	Ovary large and plump, bigger size opaque oocytes visible
IV	Maturation	>800	>8	Ovary transparent, ovaries occupy half the body cavity, transparent oocytes separable

Table 3
PCR primers used for the gene expression analyses and *in situ* hybridization (ISH).

Genes name	Primer name	Primer sequence (5′–3′)
paqr5	Forward	TTTTGACTACATCGGCCACA
	Reverse	TTTGTCACCAAGCAAAGCAG
paqr6	Forward	ATCTGCGTGTACCCCTTCAC
	Reverse	CAGAGCCACAGGGACAAAGT
paqr7a	Forward	CACATTTCTTCGGGCTATCG
	Reverse	GCTGTCGTTCACAAAGTCCA
paqr7b	Forward	TTCCCTGTACCGTCAAGCTT
	Reverse	GGGCTGGGCGTGAGGATCCCG
paqr8	Forward	AGCCAAGTCCAGATATCGCA
	Reverse	GAAGAAGAGGGCTGACGAGA
paqr9	Forward	ACAATCAGCGCCTATACCGT
	Reverse	CATTTCTGTCGGGTGTTGCA
pgr	Forward	ATGTGCCTCACATCCTGCTC
	Reverse	GCTGCCTGACTCTTCAGTCC
pgrmc1	Forward	GCTGTGAACGGGAAAGTGTT
	Reverse	ACTTAAAGGTGAACTGCTGCTC
pgrmc2	Forward	AAATCTTCGACGTGACCAGC
	Reverse	TGAACTGCATCTCCCACTCC
β-actin	Forward	GACAGGTCATCATCATTGGC
	Reverse	CAGACAGCACAGTGTTGGCATAC
ISH *paqr5*	Forward	GGGCGGGTGTTATTAACCCTCACTAAAGGG TCAACCAGGTCCCCAAAGTT
	Reverse	CCGGGGGGTGTAATACGACTCACTATAGGG ACATGGAGCTGAAGGTGTGA

Primers used for generating probe of *in situ* hybridization contain the T3 or T7 polymerase promoter sequence (underlined) at their 5′end.

examination. The fixed testes were dehydrated through a graded series of ethanol concentrations (70–100%), embedded in paraplast (Leica, Germany). The 5 μm section was obtained on a retracting microtome and stained with hematoxylin. The testicular development was classified into four phases according to GSI and analyses of histology sections: phase I (early meiosis, GSI < 0.05%, in February), phase II (mid meiosis, GSI 0.05–0.08%, in March), phase III (late meiosis, GSI 0.08–0.11%, in April), phase IV (maturation, GSI > 0.11%, in May) (Fig. 1, Table 1). The ovary was removed and the oocyte diameters were determined under a dissecting microscope. We artificially divided the ovaries into four phases according to GSI, oocyte diameters and macroscopic description: phase I (immature, GSI <2%, oocyte diameter <200 μm, in February), phase II (maturing, GSI 2–4%, oocyte diameter 200–600 μm, in March), phase III (nearly ripe, GSI 4–8%, oocyte diameter 600–800 μm, in April) and phase IV (mature, GSI >8%, oocyte diameter >800 μm, in May) (Fig. 2, Table 2).

2.6. Real-time qPCR

Specific primers for measuring the expression of target genes were designed and examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (10^3–10^8 copies/ul) (Table 3). All qPCR was performed using

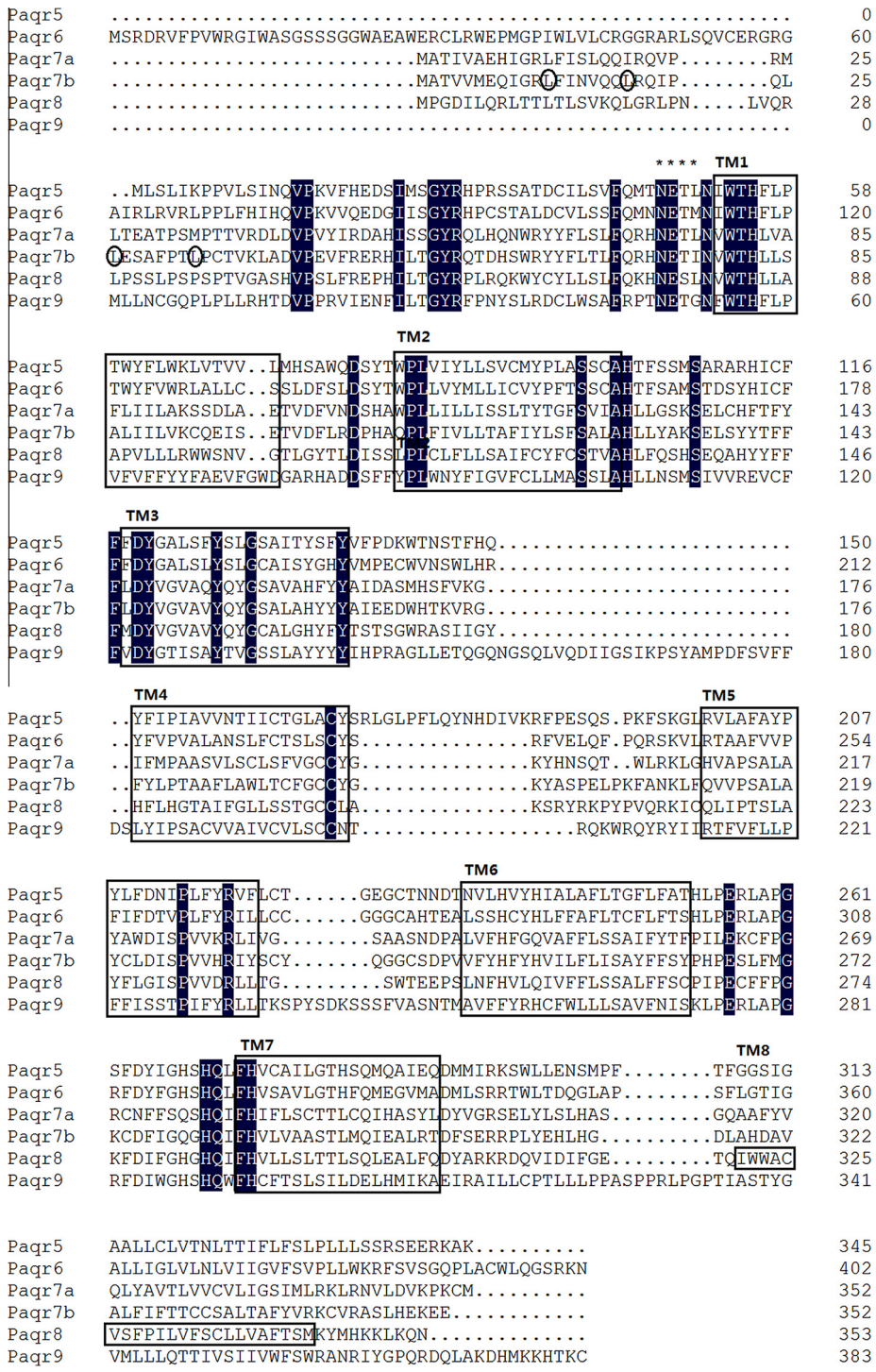

Fig. 3. Alignment of *B. sinensis* membrane progestin receptors (mPRs or Paqr) deduced amino acid sequence. Predicted seven-transmembrane domains are boxed. The conserved potential N-glycosylation site is marked with asterisks. The leucine residues in a leucine zipper motif in the N-terminus of Paqr7b are marked with circles.

premix (Fermentas). Ct values were determined in a 7500 fast real-time PCR system (Applied Biosystems, USA) using default settings and baseline, and thresholds were adjusted manually. The relative mRNA levels of the target genes were determined using the comparative Ct method (Schmittgen and Livak, 2008) with the β-actin gene used as an internal control. The specificity and efficiency of β-actin specific primers were described in our previous study (Lai et al., 2014). The absolute mRNA levels of target genes were determined based on a standard curve generated by measuring known concentrations of a serial diluted plasmid containing corresponding target gene.

2.7. Plasma DHP in female

Aliquots of plasma were separated by centrifugation at 1000*g* for 15 min at 4 °C, and stored at 80°C until analysis. Plasma DHP levels were determined using a previous protocol (Wang et al.,

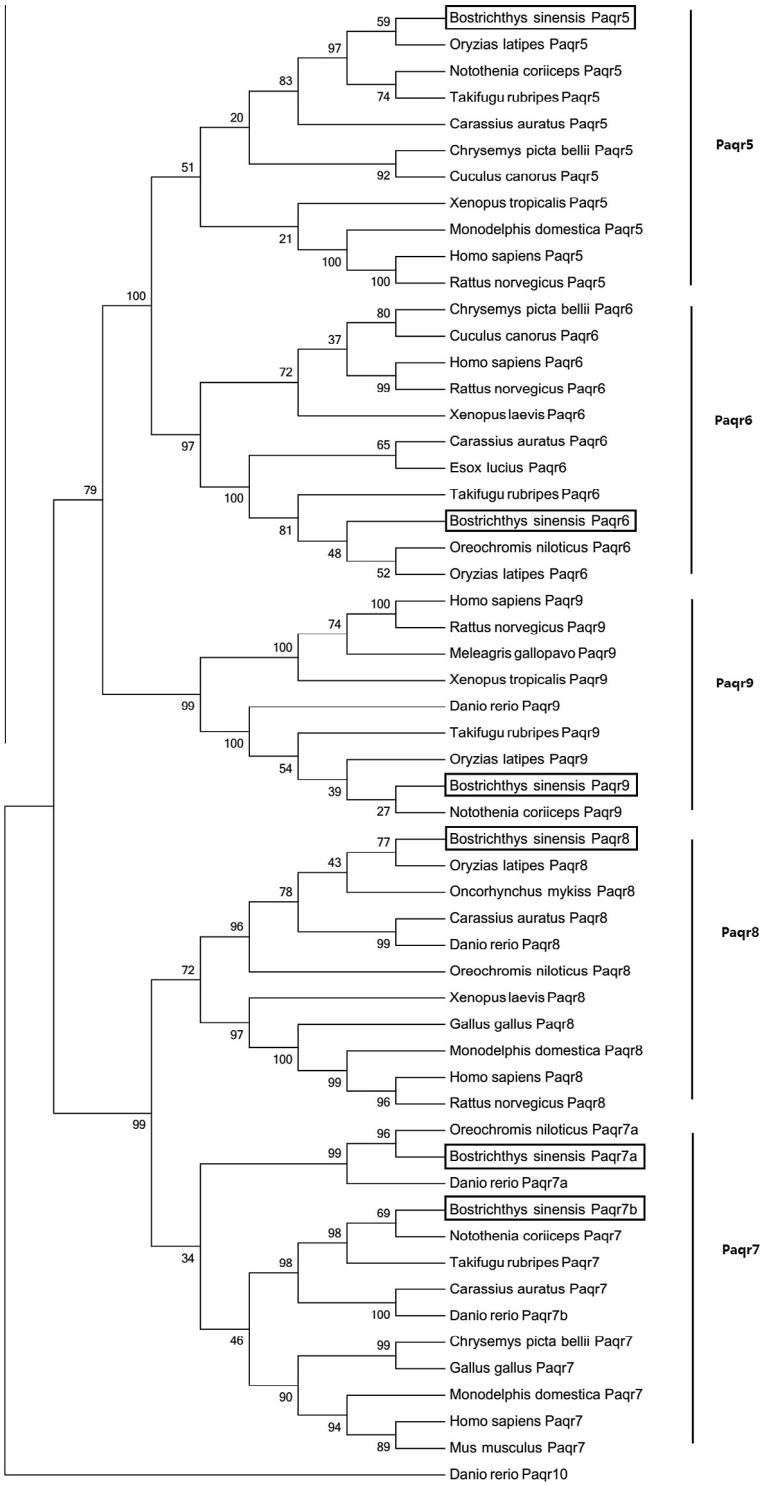

Fig. 4. Phylogenetic analysis of *B. sinensis* membrane progestin receptors (mPRs or Paqr). Multiple species' amino acid sequences of membrane progestin receptors were aligned using Clustal W. Paqr5-9 belongs to PAQR Class II, while Paqr10 belongs to PAQR Class III. Therefore, zebrafish Paqr10 protein is used as an outgroup for the tree. GenBank accession numbers for sequence data analyzed are: *Oryzias latipes* Paqr5, XP_004067005.1; *Notothenia coriiceps* Paqr5, XP_010768264.1; *Takifugu rubripes* Paqr5, XP_003969864.1; *Carassius auratus* Paqr5, BAF37035.1; *Chrysemys picta bellii* Paqr5, XP_008168729.1; *Cuculus canorus* Paqr5, XP_009565937.1; *Xenopus tropicalis* Paqr5, *Xenopus* (Silurana) *tropicalis*; *Monodelphis domestica* Paqr5, XP_007479694.1; *Homo sapiens* Paqr5, NP_060175.3; *Rattus norvegicus* Paqr5, NP_001014114.1; *Chrysemys picta bellii* Paqr6, XP_005280765.1; *Cuculus canorus* Paqr6, XP_009559566.1; *Homo sapiens* Paqr6, NP_079173.2; *Rattus norvegicus* Paqr6, NP_001178006.1; *Carassius auratus* Paqr6 (mPRγ-2), dbj|BAF37036.1; *Esox lucius* Paqr6, XP_010883641.1; *Takifugu rubripes* Paqr6, XP_003965729.2; *Oreochromis niloticus* Paqr6, ENSONIT00000010853 (Ensemble); *Oryzias latipes* Paqr6, XP_011484110.1; Homo sapiens Paqr9, NP_940906.1; Rattus norvegicus Paqr9, NP_001258081.1; *Meleagris gallopavo* Paqr9, XP_010714947.1; *Xenopus tropicalis* Paqr9, XP_004914408.1; *Danio rerio* Paqr9, XP_005166589.1; *Takifugu rubripes* Paqr9, XP_003966777.1; *Oryzias latipes* Paqr9, XP_011471733.1; *Notothenia coriiceps* Paqr9, XP_010787892.1; *Oryzias latipes* Paqr8, NP_001188424.1; *Oncorhynchus mykiss* Paqr8, NP_001118123.1; *Carassius auratus* Paqr8, BAF37034.1; *Danio rerio* Paqr8, NP_899187.1; *Oreochromis niloticus* Paqr8, ENSONIT00000004221; *Xenopus laevis* Paqr8, NP_001079330.1; *Gallus gallus* Paqr8, NP_001008462.1; *Monodelphis domestica* Paqr8, XP_001364107.2; *Homo sapiens* Paqr8, NP_588608.1; *Rattus norvegicus* Paqr8, NP_001014121.1; *Oreochromis niloticus* Paqr7a, ENSONIT00000009618; *Danio rerio* Paqr7a, NP_001093530; *Notothenia coriiceps* Paqr7, XP_010776249.1; *Takifugu rubripes* Paqr7, ABD61705.1; *Carassius auratus* Paqr7, BCE06917.1; *Danio rerio* Paqr7b, NP_899188.1; *Chrysemys picta bellii* Paqr7, XP_008169210.1; *Gallus gallus* Paqr7, NP_001157123.1; *Monodelphis domestica* Paqr7, XP_007491397.1; *Homo sapiens* Paqr7, NP_848509.1; *Mus musculus* Paqr7, NP_001272776.1; *Danio rerio* Paqr10, ADD51542.1.

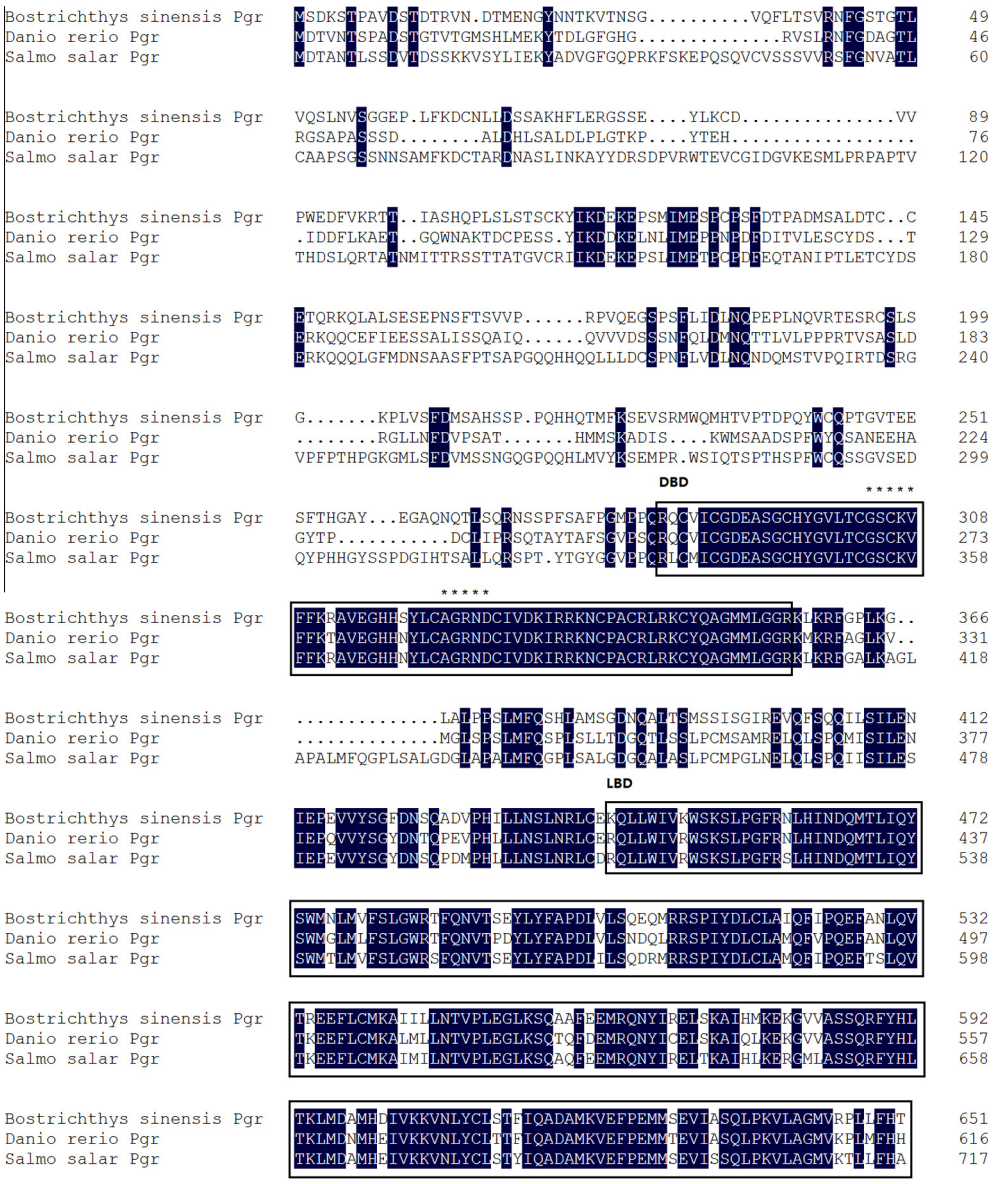

Fig. 5. Alignment of *B. sinensis* nuclear progesterone receptor (nPR or Pgr) deduced amino acid sequence. Predicted DNA binding domains (DBD) and Ligand binding domains (LBD) are boxed. Two conserved zinc finger motifs (P box (GSCKV) and the D box (AGRND)) in the DBD are marked with asterisks.

2008) and an EIA kit purchased from the Caymen Chemical Company (Ann Arbor, Michigan, USA).

2.8. Localization of paqr5 transcript in the olfactory rosette

The expression of *paqr5* mRNA in the olfactory rosette was investigated using *in situ* hybridization (ISH), which has been described previously (Chen et al., 2010). In brief, a gene-specific product was amplified by PCR using specific primers contained the T3 or T7 RNA polymerase promoter sequence attached at their 5′ends (Table 3). The PCR product (288 bp) was then gel purified and served as a template for synthesizing digoxigenin-labeled cRNA probe (Roche, Swiss). Freshly collected olfactory rosette samples were fixed in 4% w/v paraformaldehyde in PBS (pH 7.4) overnight at 4 °C, followed by immersed in 25% w/v sucrose in PBS at 4 °C until sink, and then embedded in embedding medium (Tissue-Tek™, Sakura, USA) by freezing in liquid nitrogen. Cryostat sections were cut at 10 μm thickness, and probe was added at a final concentration of 800 ng/ml. Bound DIG probes were detected with anti-DIG conjugated with POD (Roche) followed by Fluorescence (TSA™ Fluorescein System, PerkinElmer). Then the slides were washed in PBS and mounted with VectaShield containing DAPI (Vector Laboratories, Burlingame, CA, USA).

2.9. Statistical analysis

All data, presented as means ± standard error of the mean (SEM), were analyzed using one-way ANOVA followed by Fisher's PLSD post hoc test to assess statistical differences among the individual groups. The statistical analyses were run using the SPSS (version 21.0) statistical software package.

3. Results

3.1. Cloning and phylogenetic analyses of progestin receptors

Nine progesterone receptors were cloned, including *paqr5* (Genbank KT779277), *paqr6* (KT779282), *paqr7a,b* (KT156675,

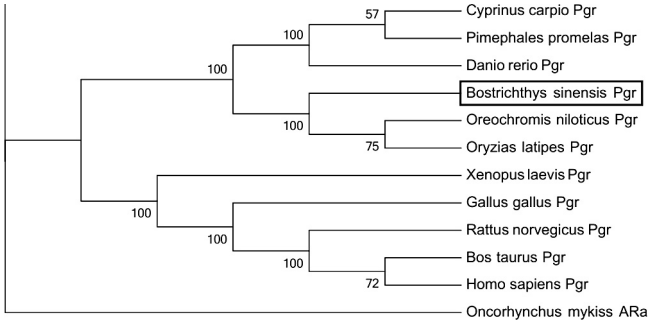

Fig. 6. Phylogenetic analysis of *B. sinensis* nuclear progesterone receptor (Pgr). Multiple species' amino acid sequences of membrane progestin receptors are aligned using Clustal W. *Oncorhynchus mykiss* androgen receptor alpha protein is used as an outgroup for the tree. GenBank accession numbers for sequence data analyzed are: *Cyprinus carpio* Pgr, BAQ02893.1; *Pimephales promelas* Pgr, AFM74474.1; *Danio rerio* Pgr, NP_001159807.1; *Oreochromis niloticus* Pgr, AIE56465.1; *Oryzias latipes* Pgr, NP_001165515.1; *Xenopus laevis* Pgr, NP_001079100.1; *Gallus gallus* Pgr, NP_990593.1; *Rattus norvegicus* Pgr, NP_074038.1; *Bos taurus* Pgr, NP_001192285.1; *Homo sapiens* Pgr, AAA60081.1; *Oncorhynchus mykiss* ARa, NP_001117656.1.

KT779275), *paqr8* (KT779276), *paqr9* (KT779278), *pgr* (KT779279), *pgrmc1,2* (KT779280, KT779281). The cDNAs of the *paqr5*, *6*, *7a*, *7b*, *8* and *9* encoded proteins of 346, 404, 353, 353, 354 and 385 amino acid residues, respectively. The *B. sinensis* mPRs share high sequence identity with those of other fish species (55–81%) and relatively lower homology (37–57%) with their mammalian counterparts. A potential N-glycosylation site (N{P}S/T{P}) preceding transmembrane (TM) 1 was found in all *B. sinensis* mPR subtypes (Fig. 3). The *B. sinensis* Paqr5, 6, 7b, 8 and 9 contained seven transmembrane domains, characteristics of G protein-coupled receptors, based on hydrophobicity analysis (DAS-domain prediction) (Ashley et al., 2006). Although Paqr7a protein was predicted as possessing five transmembrane domains, computer analysis by TMHMM program predicted that it had seven transmembrane domains. Moreover, the Paqr8 had an eighth predicted TM domain at C-terminus by TMHMM program. A leucine zipper pattern (L{6}L{6}L{6}L) was found in the N-terminus of Paqr7b, which could contribute to the formation of homodimers or heterodimers with other proteins. Phylogenetic analyses classified these mPRs into five distinct subtypes (Fig. 4). Each form of *B. sinensis* mPRs was classified (or grouped) together with their respective mPR subtype groups. The CDS of *pgr* consisted of 1959 nucleotides, encoding a protein of 652 amino acids and the ligand-binding domain showed high homology with Pgr of other vertebrates (Fig. 5). The phylogenetic tree showed *B. sinensis* Pgr was classified into the fish clade (Fig. 6). The deduced Pgrmc1 and Pgrmc2 proteins were 183 and 200 amino acid long and both of them contained a transmembrane domain at N-terminus (Fig. 7). *B. sinensis* Pgrmc1 protein contained a sequence highly related to the leucine zipper pattern (L{6}L{6}L{7}L{6}L) (26–56 residues), but it is not conserved among the other Pgrmc1 proteins identified so far. In addition, a cytochrome b5 like heme/steroid binding (cyt-b5) domain was found at positions 64–161 for Pgrmc1 and 80–177 for Pgrmc2, using Conserved Domain Search (CD-search). The phylogenetic analyses showed that *B. sinensis* Pgrmc1 and Pgrmc2 were clearly associated with teleost Pgrmc1 and 2, respectively (Fig. 8).

3.2. Tissue specific expression of the progestin receptors

The tissue specific expressions of each progestin receptors were similar between male and female (Fig. 9). Expressions of *paqr*5 and *paqr8* were considerably restricted, and were undetectable in brain (Fig. 9A, E). The *paqr5* transcripts were only found in the olfactory rosette, gill, skin and liver, and the highest level was expressed in the olfactory rosette (Fig. 9A). Interestingly, these tissues, except liver, are in direct contact with external environment. In addition to expressions in the olfactory rosette, gill, skin and liver, the *paqr8* was also expressed in gonad and intestine. Expressions of *paqr6*, *paqr7a*, *paqr7b* and *paqr9* were found in all the tissues examined, and they were abundantly expressed at different levels in different areas of the brain (Fig.9B, C, D, F). For example, the mesencephalon expressed more *paqr9*, but less *paqr7a* and *b* than the telencephalon. The testicular and ovarian tissues expressed the highest *pgr* transcript levels (Fig. 9G). In addition, *pgr* mRNAs were also found in other tissues examined, even though their transcript levels were much lower than that of gonad. The transcripts of *pgrmc1* and *pgrmc2* were detectable in all the tissues examined (Fig. 9H, I). The liver expressed the highest transcript level of *pgrmc1*, while the skin expressed the highest transcript level of *pgrmc2*. Importantly, all the progestin receptors were expressed in the olfactory rosette, indicating they are involved in deciphering progestin signaling in the olfactory system.

3.3. Expressions of progestin receptor mRNAs in the olfactory rosette during the reproductive cycle

3.3.1. In male

We determined the expression levels of mPRs, *pgr*, *pgrmc1* and *pgrmc2* in the olfactory rosette during the testicular development. GSI increased significantly from phase I to phase IV, and the highest value was observed at phase IV (GSI 0.22%) when testes were full of spermatozoa (Fig. 1). The expression levels of *paqr6*, *paqr9* and *pgrmc1* fluctuated, but did not show significant

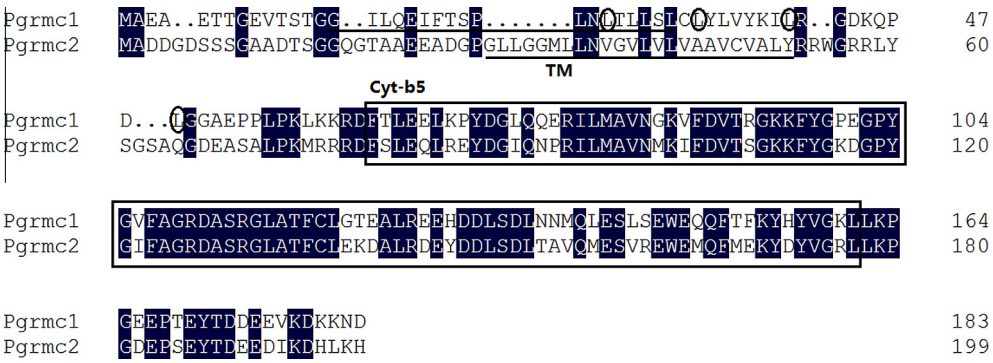

Fig. 7. Alignment of *B. sinensis* progesterone receptor membrane component (Pgrmc) deduced amino acid sequence. Predicted cytochrome b5 like heme/steroid binding (cyt-b5) domains are boxed. The leucine residues in a leucine zipper motif of Pgrmc1 are marked with circles. Predicted transmembrane domains are underlined.

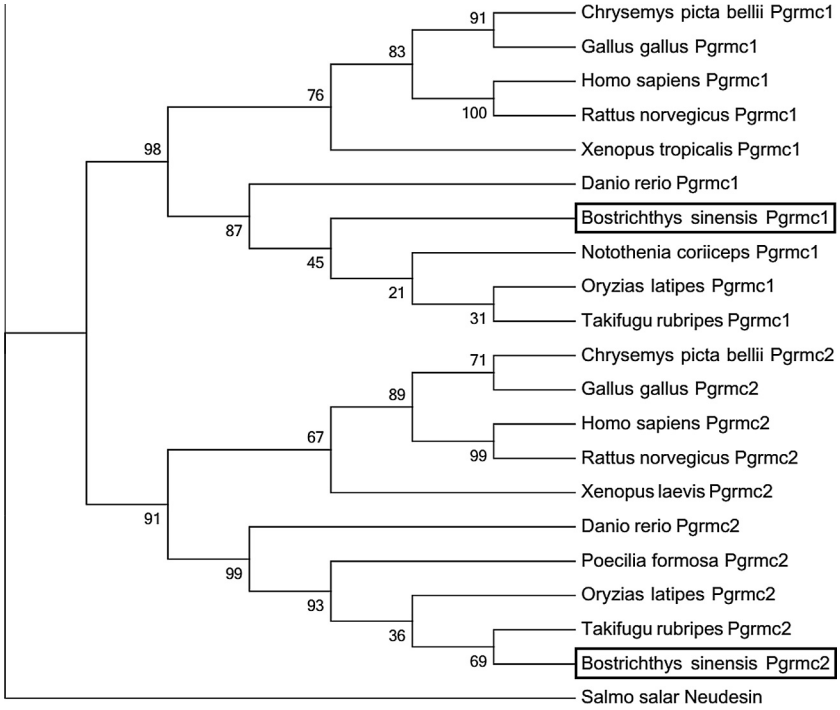

Fig. 8. Phylogenetic analysis of *B. sinensis* progesterone receptor membrane component (Pgrmc). Multiple species' amino acid sequences of membrane progestin receptors were aligned using Clustal W. *Salmo salar* Neudesin protein is used as an outgroup for the tree. GenBank accession numbers for sequence data analyzed are: *Chrysemys picta bellii* Pgrmc1, XP_005299021.1; *Gallus gallus* Pgrmc1, NP_001258868.1; *Homo sapiens* Pgrmc1, CAG33274.1; *Rattus norvegicus* Pgrmc1, AAH62073.1; *Xenopus tropicalis* Pgrmc1, NP_001006842.1; *Oryzias latipes* Pgrnc1, NP_001098572.1; *Danio rerio* Pgrmc1, NP_001007393.1; *Takifugu rubripes* Pgrmc1, XP_003970860.1; *Notothenia coriiceps* Pgrmc1, XP_010792871.1; *Chrysemys picta bellii* Pgrmc2, XP_005282812.1; *Gallus gallus* Pgrmc2, NP_001006441.1; *Homo sapiens* Pgrmc2, NP_006311.2; *Rattus norvegicus* Pgrmc2, NP_001008375.1; *Xenopus laevis* Pgrmc2, NP_001087737.1; *Danio rerio* Pgrmc2, NP_998269.1; *Oryzias latipes* Pgrmc2, NP_001098199.1; *Poecilia formosa* Pgrmc2, XP_007568664.1; *Takifugu rubripes* Pgrmc2, XP_003962882.1; *Salmo salar* Neudesin, NP_001134765.

differences from the phase I to phase IV (Fig. 10). In contrast, the expression levels of other progestin receptors exhibited significant differences. Expression levels of *paqr7a* and *paqr7b*, *pgr* and *pgrmc2* mRNAs were relatively low at phase I, and reached their peak levels at phase II. Thereafter, a significant decrease of *pgr*, and slight but not significant decreases of *paqr7a* and *paqr7b* were found, while *pgrmc2* expression maintained at relatively high levels at phases III and IV. Interestingly, like *paqr7a* and *paqr7b*, the lowest level of *paqr8* expression was observed in phase I. However, up-regulation of *paqr8* mRNA began at phase III. Then it slightly declined at phase IV (Fig. 10E). The level of *paqr5* was high at phases I and II, and then decreased gradually at phases III and IV, which was negatively correlated with GSI change (Fig. 10A). It is worth mentioning that the most abundant transcript among progestin receptors in the olfactory rosette was *pgrmc2* (>100,000 copies per 100 ng total RNA), followed by *paqr5* (about 40,000 copies per 100 ng total RNA), which indicated their dominant function in the olfactory rosette.

3.3.2. In female

In the females, GSI and oocyte diameter continuously increased from phase I to phase IV, and reached their peaks at phase IV, when ovaries contained full grown oocyte (Fig. 2). The changes of progestin receptor expressions in the females were different from those in males in the olfactory rosette. The *paqr5*, *paqr6*, *paqr7a* *paqr7b*, *paqr8*, *paqr9*, *pgrmc1* and *pgrmc2* mRNAs expressions varied slightly, but were not significant from phases I–IV (Fig. 11). The transcript levels of *pgr* were highest at phases I and II, declined at phase III and dropped to its lowest value at phase IV (Fig. 11G).

3.4. Plasma DHP concentrations in female

Plasma DHP concentrations in female were relatively low in February (phase I) and March (phase II), and reached its peak in April (phase III). Thereafter, a slight decrease was found from April to May (phase III to phase IV) (Fig. 12). The plasma DHP levels in female revealed a similar trend to the expression changes of *paqr8* in male olfactory rosette during the reproductive cycle, the level of which also peaked at phase III (April) (Fig. 10E). It is likely that Paqr8 is a candidate for male to detect DHP signaling released by female.

3.5. Cellular localization of paqr5 mRNA expression in the olfactory rosette

The *B. sinensis* olfactory rosette that is fusiform contains about 10–16 primary olfactory lamellae. Each lamella is composed of olfactory epithelium that contains many layers of cells, including sensory and non-sensory cells, and central core that consists of collagenous fiber, reticular fiber and blood capillary (Ma et al., 2003). In the present study, a clear ISH signal was observed in the intermediate and basal part of the olfactory epithelium, while almost no signal was present in the central core or the apical part of the olfactory epithelium (Fig. 13). Interestingly, some microvillous or ciliated sensory neurons like cells were also found to express *paqr*5 transcripts (Fig. 13G–I).

4. Discussion

4.1. Cloning and phylogenetic analysis of progestin receptors

Among all the *B. sinensis* mPRs, only the Paqr8 had the eighth predicted TM domain using TMHMM program. The presence of this predicted TM domain at the C-terminus is found in all the human mPR subtypes and it is diagnostic of progesterone receptors among PAQR family (Smith et al., 2008). In contrast, the predicted eighth TM domain is not found in goldfish mPR subtypes (Paqr5, 6, 7, 8)

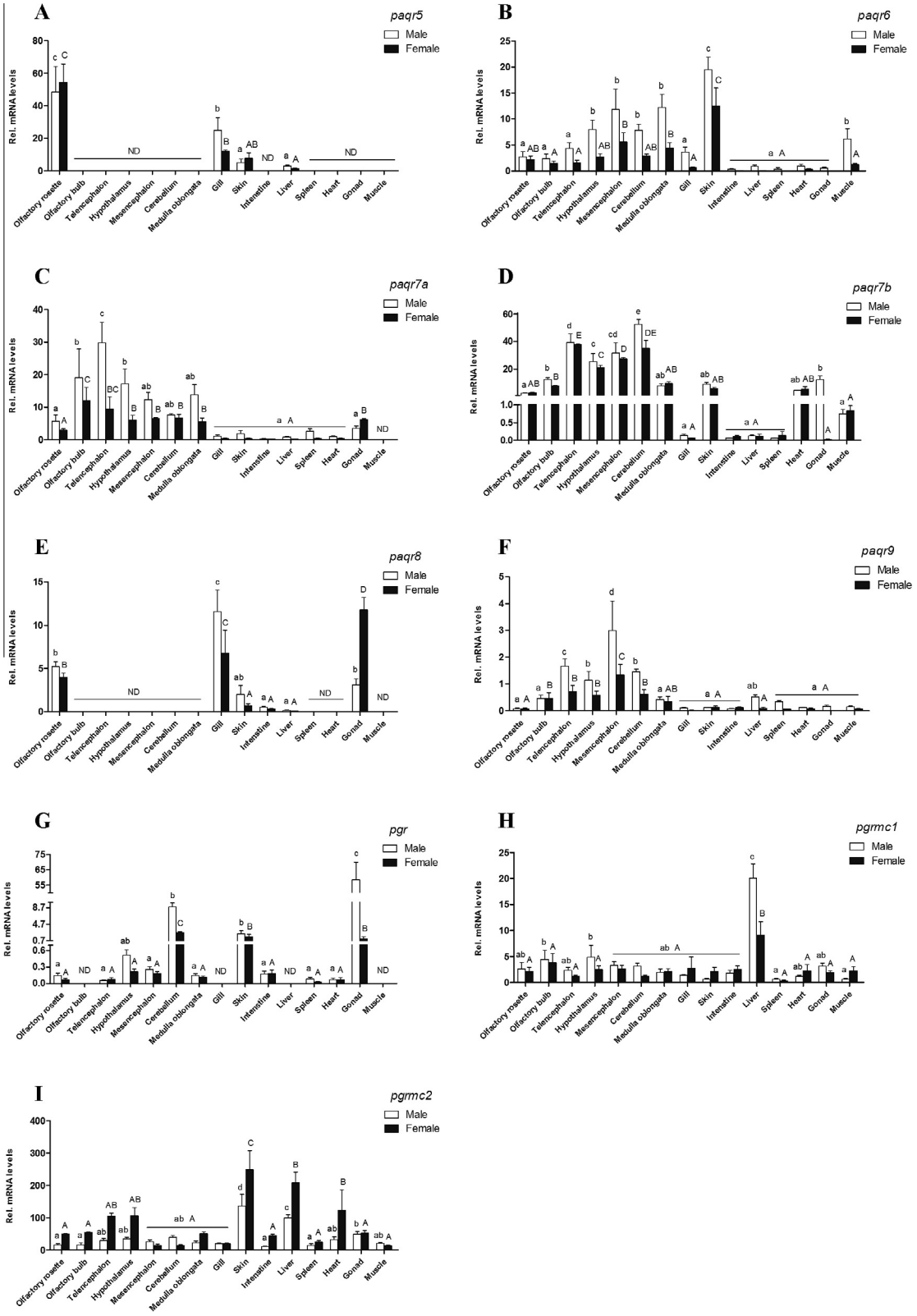

Fig. 9. Tissue specific expression of progestin receptors in *B. sinensis*. The levels of the respective mRNAs were determined using qPCR and normalized to the internal housekeeping gene (β-actin). Data are expressed as the mean ± SEM (n = 4). Bars marked with different letters (small letters for male, capital letters for female) are significantly different from each other ($p < 0.05$). ND, not detectable.

(Tokumoto et al., 2012). Although the predicted eighth TM may place the C-terminus of mPRs in (or out) the cytoplasm, no evidence shows it is required for either progesterone sensing or downstream signal transduction (Smith et al., 2008).

Two distinct *pgr* genes have been reported in the eel and *Xenopus* (Bayaa et al., 2000; Tian et al., 2000; Todo et al., 2000; Ikeuchi et al., 2002), while only one *pgr* gene was found in zebrafish, medaka, Takifugu and salmon (Chen et al., 2010, 2011;

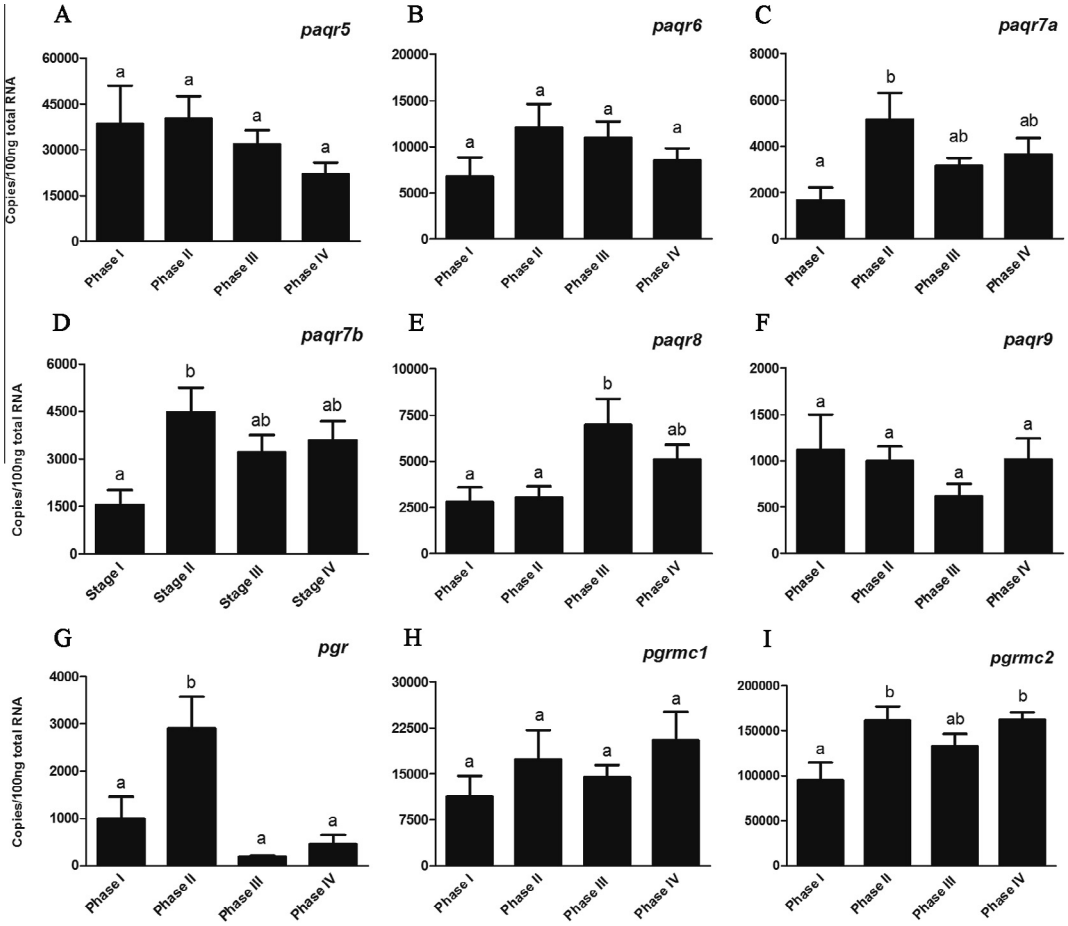

Fig. 10. Expression of progestin receptors in the olfactory rosette of male *B. sinensis* during the reproductive cycle. Phase I (Early meiosis), Phase II (Mid meiosis), Phase III (Late meiosis), Phase IV (Maturation). Data are expressed as the mean ± SEM (n = 6). Bars marked with different letters are significantly different from each other ($p < 0.05$).

Hanna et al., 2010). Experimental trials to isolate additional *pgr* cDNAs or transcriptomic data obtained from the brain and olfactory rosette samples did not provide any evidence for the existence of additional *pgr*-like genes or mRNA isoforms in *B. sinensis*. Two Pgr genes are likely eel and *Xenopus* specific.

Thomas et al. (2014) reported a close association of human Pgrmc1 with mPRα (Paqr7) in cell membrane and suggested that the coupling of Pgrmc1 to mPRα involves the heme binding domain of Pgrmc1. Interestingly, a leucine zipper pattern (L{6}L{6}L{6}L) and a sequence highly related to the leucine zipper pattern (L{6}L{6}L{7}L{6}L) were found in the N-terminus of *B. sinensis* Paqr7b and Pgrmc1 respectively. These sequences could contribute to the formation of homodimers or heterodimers between *B. sinensis* Paqr7b and Pgrmc1.

4.2. Tissue distribution

The tissue-specific expressions of all nine progestin receptors were similar between male and female in *B. sinensis*. The *paqr5* showed different tissue-specific expression in different teleost species. In catfish *Ictalurus punctatus*, the predominant expressions of *paqr5* were found in the kidney and the intestinal tissues, which is similar to the results found in humans (Kazeto et al., 2005; Tang et al., 2005). In contrast, *paqr5* was undetectable in the kidney and intestine in goldfish (Tokumoto et al., 2012). Interestingly, *paqr5* transcripts were expressed in gills in both catfish and goldfish, suggesting that Paqr5 may be involved in ion regulation (Kazeto et al., 2005). In the present study, *paqr5* transcript was found in the olfactory rosette, gill, skin and liver (Fig. 9A). These organs except liver are in direct contact with external water, which suggests the function of Paqr5 is likely to be involved in detecting external signaling including progestin.

In the present study, *paqr8* was expressed abundantly in gonadal tissues in *B. sinensis*, which is consistent with other teleost species, such as goldfish (Tokumoto et al., 2012), catfish (Kazeto et al., 2005) and rainbow trout (Mourot et al., 2006). Zebrafish Paqr8 protein immunoreactions were found in scattered cells in the pituitary, which suggests it may be involved in progestins mediated rapid release of gonadotropin and subsequent downstream signaling (Hanna and Zhu, 2009). However, *paqr8* transcript was undetectable in the brain of *B. sinensis*. It is likely that other progestin receptors, such as Paqr9 that is primarily expressed in the brains of *B. sinensis* and in human pituitary, mediate rapid progestin signaling in brain in *B. sinensis*.

In mammals, Pgr has a wide range of tissue expression and is detected in uterus, ovary, vagina, testis, breast, brain, vascular endothelium, thymus, pancreatic islet, osteoblast like cells and lung (Graham and Clarke, 1997). Similarly in teleost, *pgr* is primarily expressed in reproductive organs and is also detectable in brain and most periphery tissues (Chen et al., 2010, 2011). In addition to dominant expressions of *pgr* found in gonads, cerebellum and skin, *pgr* was also expressed in most tissues examined including hypothalamus, heart, intestine, spleen and olfactory rosette, suggesting extended physiological functions of progestin besides reproduction in *B. sinensis*. For example, progesterone and allopregnanolone exert several effects in brain including

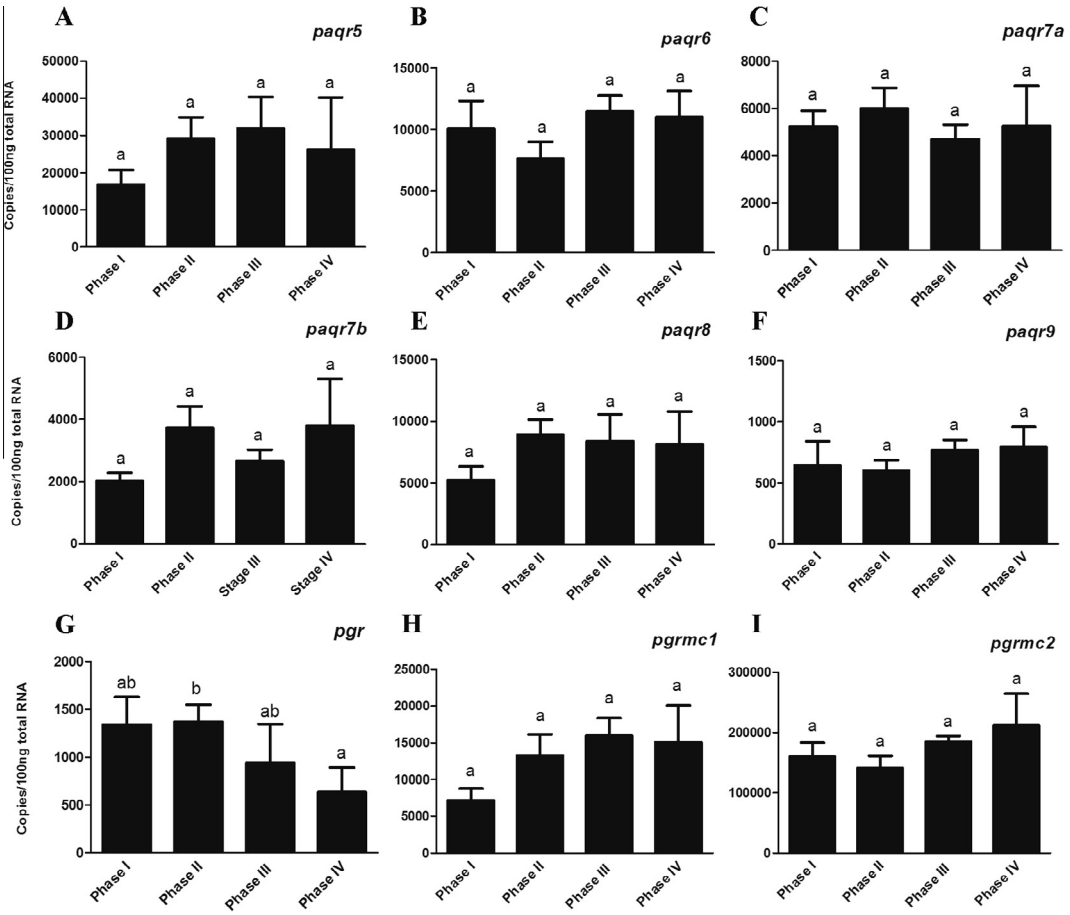

Fig. 11. Expression of progestin receptors in the olfactory rosette of female *B. sinensis* during the reproductive cycle. Phase I (Immature), Phase II (Maturing), Phase III (Nearly ripe) and Phase IV (Maturation). Data are expressed as the mean ± SEM (n = 6). Bars marked with different letters are significantly different from each other ($p < 0.05$).

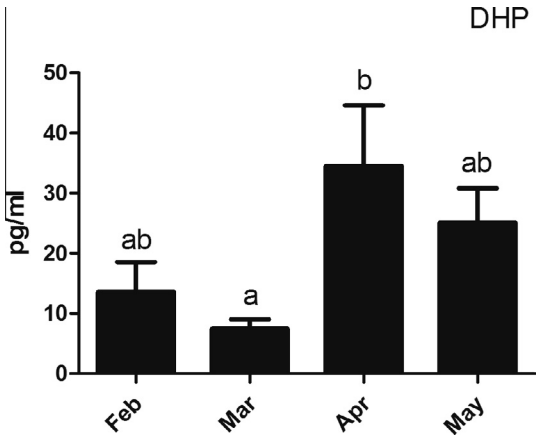

Fig. 12. Plasma DHP levels in female Chinese black sleeper *B. sinensis* during the reproductive cycle. Data are expressed as the mean ± SEM (n = 6). Bars marked with different letters are significantly different from each other ($p < 0.05$).

neuroprotection and neurogenesis in mammalians (Intlekofer and Petersen, 2011; Guennoun et al., 2015).

Paqr6, *paqr7a*, *paqr7b*, *paqr9*, *pgrmc1* and *pgrmc2* were expressed in a wide range of tissues in *B. sinensis*, suggesting that they cooperatively mediate multiple non-genomic progestin actions in many target tissues. Importantly, all the progestin receptors were expressed in the olfactory rosette. Based on the absolute quantification of the nine progestin receptors mRNAs in the male olfactory rosette during the reproductive cycle (Fig. 10), the most abundant receptor in male olfactory rosette was *pgrmc2*, followed by *paqr5*, then *pgmrc1*, *paqr6*, *paqr7a*, *paqr7b*, *paqr8*, *pgr* and *paqr9* in order. In addition to a wide tissue distribution of *pgrmc2*, *pgrmc2* also showed relative high expression levels in the hypothalamus, heart, skin, and liver compared to relatively lower expression in the olfactory rosette (Fig. 9I), suggesting Pgrmc2 may play key roles other than pheromone signaling in these tissues. In contrast, the *paqr5* was found to be the most abundant in the olfactory rosette compared to other tissues. In addition, the expression changes of *paqr8* in male olfactory rosette were correlated with the changes of plasma DHP concentrations in females. It is possible that the *paqr*5 and *paqr*8 play important roles in the detection of DHP signaling released from female fish in the olfactory rosette in male fish, but it is also possible that composition and changes of each progestin receptor in the olfactory rosette are important for detecting an array of progestins in a complex environment.

4.3. Expression changes of progestin receptors during the reproductive cycle

4.3.1. In male

In goldfish, DHP is a pre-ovulated pheromone. Postvitellogenic female goldfish exhibit a dramatic preovulatory LH surge when exposed to appropriate stimuli, and plasma DHP also increases dramatically during the middle portion of the LH surge (Kobayashi et al., 2002). DHP not only promotes the germinal vesicle breakdown of oocyte and subsequent ovulation, but also is released through gill into water as a pheromone (Kobayashi et al., 2002).

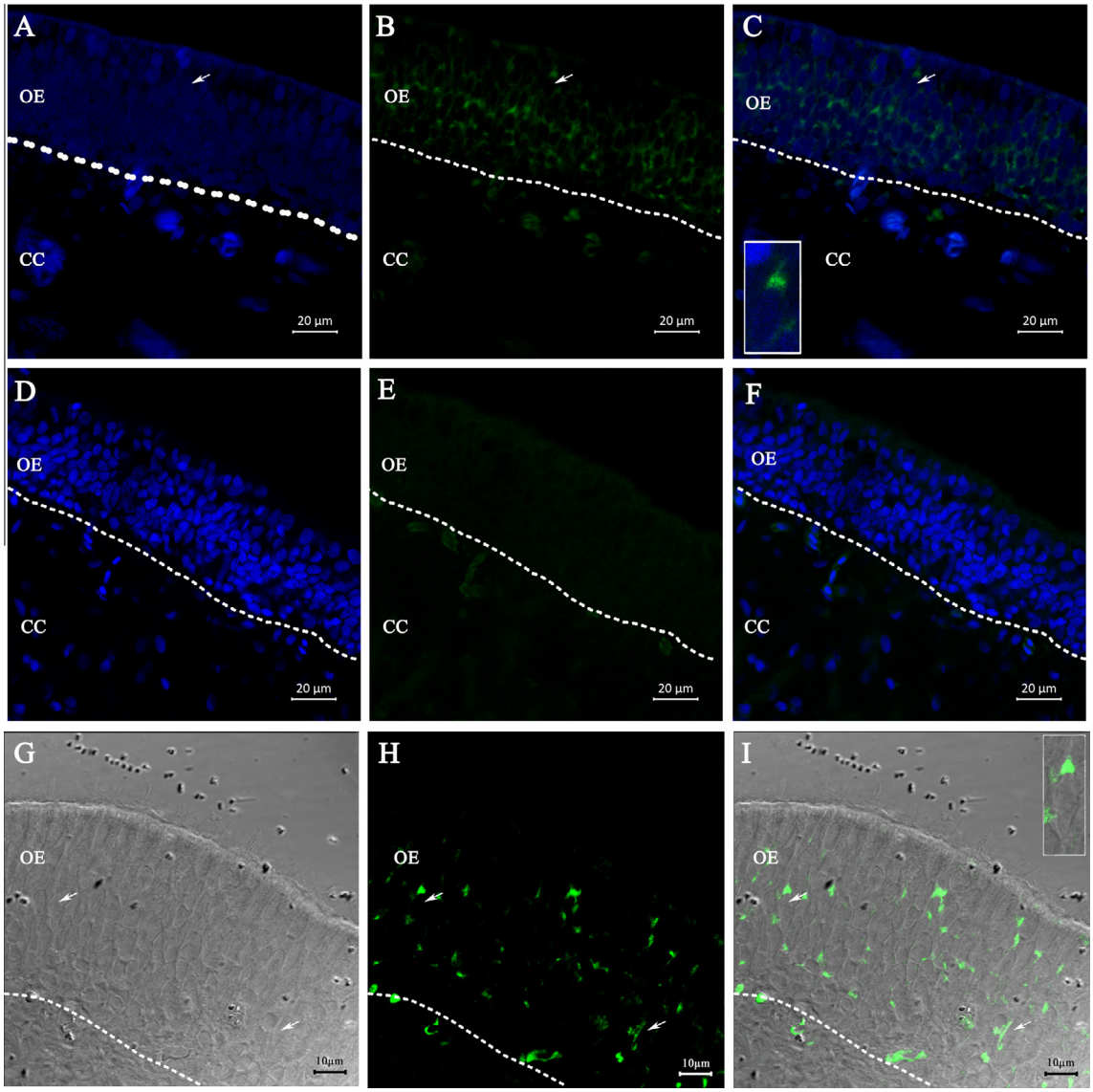

Fig. 13. Cellular localization analysis of *paqr*5 mRNA expression in the olfactory rosette using confocal microscope imaging. Top panels (A-C) show representative images of same area of a section stained with nuclear specific dye DAPI (A) or *paqr*5 antisense probe (B), and a merged image (C). Middle panels (D–F) show representative images of a section stained with DAPI (D), or a sense control riboprobe for *paqr*5 (E), and a merged image (F). Bottom panels (G–I) show a representative Differential Interference Contrast (DIC) image (G) of same cells reacted with *paqr*5 antisense probe (H), and a merge image (I). Transcripts of *paqr*5 expressed in cytoplasm of cells are located in the intermediate and basal part of the olfactory epithelium. The insets in panels C and I are magnified images of a single cell with microvillous neuron-like shape. White arrows indicate putative sensory neuron. Dashed lines mark the basal lamina. OE, olfactory epithelium; CC, central core.

Males benefit from greater olfactory sensitivity to DHP when the female are ready to spawn. The levels of olfactory sensitivities may be modulated by changes in the types, numbers of the receptors and/or the sensitivities of these receptors (Creese and Sibley 1981; Habibi et al. 1989). In the present study, the plasma DHP level of female *B. sinensis* peaked in April when the testes of male fish were at phase III. Among the nine progestin receptors, only *paqr8* expression level increased with the development of testis and peaked at phase III when the male are ready to spawn, suggesting the *paqr8* plays an important role in sensing DHP than others. The expression level of *paqr5* was the highest in the olfactory rosette among the tissues examined, it is expected that the *paqr5* should be the most important progestin receptor in the olfactory rosette. Unexpectedly, a slight but not significant decrease of *paqr5* expression was found in the olfactory rosette during the maturation. It is possible that the function of highly expressed *paqr5* before the maturation is to suppress the olfactory rosette to detect sex pheromone DHP by competing for the ligands and mediating different signaling transduction with other membrane progestin receptors (e.g. *paqr8*). With the reproductive readiness of males, the expression of *paqr7a* and *paqr7b* at phase II and *paqr8* at phase III reached their peaks when *paqr5* started to decrease. At this point, these relatively low expressed receptors have more chance to bind limited ligands in external environment and exert specific signaling transduction. Alternatively, the decrease of *paqr*5 and other progestin receptor transcripts may reflect utilization of transcripts for the translation, and therefore increase the protein expression, receptor amount, and sensitivity, which implicate their important roles in detecting pheromone signaling. In the present study, the expression of progestin receptors was only determined at mRNA level. It is known that complicated biological processes, such as transcriptional splicing, post-transcriptional splicing, translational modifications, translational regulation, and protein complex formation, might affect

the relative quantities of mRNA and protein of various genes to various degrees (Guo et al., 2008). Therefore, future studies require development of specific antibodies for each progestin receptor, and correlate changes of transcripts with changes of proteins and physiological status.

4.3.2. In female

In goldfish, waterborne DHP appears to act as a 'female' pheromone to induce ovulation among other female fish (Kobayashi et al., 2002). Female goldfish olfactory system detects sex steroids as well as males (Sorensen and Stacey, 1987), and females experienced higher ovulation rates in the absence of spawning substrate when exposed to DHP overnight (Sorensen et al., 1987). The olfactory sensitivities of female and male *B. sinensis* to sex pheromones were related to their gonadal maturity, but the relativity of the female was lower than that of the male (Ma et al., 2003). In this study, we found that the expression changes of *paqr5*, *paqr7a*, *paqr7b*, *paqr8* and *pgr* in female were different from those in male. The expressions of *paqr5*, *paqr7a*, *paqr7b* and *paqr8* varied insignificantly throughout all phases in female. These results indicate that the mechanisms of DHP detection are different between the female and male.

Interestingly, *pgr* levels were lower in the olfactory rosette at the mature stage than those at the immature stage in both females and males. 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). Since this process is too rapid to be explained by relatively low-acting genomic steroid signaling mechanisms, in which responses occur over-time scale of hours to days, it is possible that the main function of Pgr in the olfactory rosette was not to detect the pheromone DHP. Maruska and Fernald (2010) reported that reproductive gravid female *Astatotilapia burtoni* has lower mRNA levels of androgen receptors and estrogen receptors in the olfactory bulb compared to both mouth brooding and recovering females and suggested that this change may simply be a homeostatic mechanism to maintain constant hormone sensitivity of the olfactory bulbs as the circulating hormone levels fluctuate across the reproductive cycle. Moreover, steroids like androgen can alter the olfactory sensitivity to sex pheromone in cyprinids (Cardwell et al., 1995; Belanger et al., 2010). Since the function of progestin receptors in the olfactory rosette for sensing circulating progestin cannot be ruled out, increased circulating progestin may alter the expression of some pheromone receptors including Pgr in the olfactory rosette.

4.4. Cellular localization of paqr5 mRNA in the olfactory rosette

In teleost, in addition to non-sensory cells such as basal cells and supporting cells, three morphologically distinct types of sensory cells are present in the olfactory epithelium (Døving, 2007). They can be distinguished by their characteristic shape and spatial position in the olfactory epithelium: a slender dendrite and a basal soma for ciliated neurons, a plump cell body and an intermediate soma position for microvillous neurons, and a large globose soma with an apical position for crypt neurons (Bazáes et al., 2013). Moreover, it is believed that the ciliated neurons are tuned towards social signals, the microvillous neurons detect food odorants, and the crypt neurons are selective for odorants related to reproduction (Bazáes et al., 2013). *B. sinensis paqr5* mRNA was present in the intermediate and basal part of the olfactory epithelium, suggesting that some microvillous and ciliated neurons may express *paqr5* mRNA and take part in sensing external progestin (Fig.13). In *Salmo trutta fario*, the Pgr immunoreactive cellular bodies are found in the basal region of the olfactory epithelium with long dendrites to the surface (Varricchio et al., 2010). It is likely that some sensory cells situated deep in the olfactory epithelium participate in the detection of external progestin. Locating other progestin receptors and identifying the types of cells that express *paqr5* mRNA in the olfactory rosette will be important subjects in further studies.

In summary, we first cloned the cDNAs of the nine progestin receptors (*paqr5*, *6*, *7*(*a*, *b*), *8*, *9*, *pgr*, *pgrmc1* and *pgrmc 2*) in *B. sinensis*. Some of these genes showed restricted tissue expression such as *paqr*5 and *paqr*8, whereas all of these progestin receptors were found in the olfactory rosette with various quantities. The *paqr*5 expression was further localized in cells that are located in the intermediate and basal part of the olfactory epithelium. Furthermore, different expression changes of each progestin receptor during the reproductive cycle in male and female olfactory rosette were observed. Taken together, these nine progestin receptors may be all involved in the detection of progestin in the olfactory rosette. This study provides the first evidence on the changes of all purported progestin receptors during a reproductive cycle in teleost olfactory rosette, and suggests that distinct olfactory sensitivities to DHP may be due to the changes and compositions of each progestin receptor in *B. sinensis*.

Acknowledgments

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