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Changes of melatonin and its receptors in synchronizing turbot (*Scophthalmus maximus*) seasonal reproduction and maturation rhythm

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Abstract

In most fish, reproduction is seasonal or periodic under the suitable conditions. In turbot (*Scophthalmus maximus*) farms, one of the most economically important marine flatfish species, changes in daylength could cause changes in the spawning time. In this study, to characterize the regulation of reproductive physiology following light signals, three melatonin receptors (*Mtnr*) investigated in turbot were named *smMtnr1*, *smMtnr2*, and *smMtnr1c*. Distinct expression profiles demonstrated that *Mtnr* mRNAs were concentrated in the brain (as detected in the hypothalamus (Hy) and mesencephalon (Me)), gonad and eye. The most abundant *Mtnr1* and *Mtnr2* mRNA expression levels were detected in the central nervous system at the beginning of the breeding season, suggesting that *Mtnr1* and *Mtnr2* may play vital roles in the regulation of turbot gonadal development. In addition, the melatonin profiles gradually increased and reached to the highest level at the spawning stage, indicating that melatonin is a potent hormone in the regulation of fish oocyte growth and maturation. The results of this study suggested that melatonin is the primary factor that transduces the light signal and regulates the physiological functions of turbot seasonal reproduction. Moreover, the results of this study may establish a foundation for further research seeking to identify fish melatonin receptors involved in the gonadal development and gamete maturation.

Key words: turbot, brain, melatonin, melatonin receptors, seasonal reproductive development

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1 Introduction

Reproduction is the most important process for all organisms. To obtain more offspring, reproductive behavior generally occurs in a particular season in which the natural environmental conditions are suitable. In some fish, reproduction is seasonal or periodic for suitable conditions. Many studies have shown that the factors of photoperiod and temperature regulate the seasonal occurrence of reproduction (Choi et al., 2015; Maitra and Hasan, 2016). Indeed, through artificial changes in photoperiod, the reproduction cycles in fish farms may be controlled in many species, such as tilapia (Kim et al., 2018; Rad et al., 2006).

Reproductive physiology is considered to be controlled by neuroendocrine regulation, which encompasses the brain and the endocrine system (Rocha et al., 2013). The neuroendocrine system acts in response to changing environmental factors (Nishiwaki-Ohkawa and Yoshimura, 2016; Plant, 2015). Recently, an increasing number of studies have demonstrated that artificial light or photoperiod affect reproduction in mammals, as well

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as in birds and fish, and changes in photoperiod could cause the breeding season to occur earlier or later (Choi et al., 2015; Robert et al., 2015; Rocha et al., 2013). In fish, light-sensory systems are located in the pineal organ and the eyes, and can transduce light signals to physiological signals reaching the hypothalamus–pituitary–gonadal (HPG) axis (Boeuf and Le Bail, 1999; Hastings et al., 1987; Plant, 2015). On the other hand, melatonin is synthesized in the pineal organ, the organism maintains a low melatonin concentration in the daytime, and the melatonin concentration gradually increases at night (Hastings et al., 1987). Thus, the optical environment clearly regulates melatonin production to rhythmically affect reproduction.

Melatonin (N-acetyl-5-methoxytryptamine), an essential hormone that synchronizes daily and annual variations in photoperiod and neuroendocrine function, belongs to the pertussis toxin-sensitive G-protein (guanine nucleotide-binding protein) family and has high affinity for its receptors. In the past several years, researchers have verified that melatonin plays a role in the HPG axis through specific melatonin receptors (MTs) belonging to the G-protein-coupled receptor superfamily. Generally, there are three different subtypes of MTs in fish: MT1, MT2, and MT3 (Mel1c). MTs have been detected in the retina and distinct brain areas using 2-[^{125}I]-iodo-melatonin (^{125}IMel) binding and *in situ* hybridization analysis in various fish species (Davies et al., 1994; Falcón et al., 2010; Patiño et al., 2008; Park et al., 2007a). Furthermore, the expression of MT mRNA has also been reported in other tissues, including kidney, intestine, liver, gills, muscle and skin (Kim et al., 2018; Kulczykowska et al., 2006; Park et al., 2007b; Sauzet et al., 2008). Meanwhile, MT mRNA increased in neural tissues at night under both light–dark (LD) and constant dark (DD) conditions in olive flounder (*Paralichthys olivaceus*) (Shin et al., 2011). MTs were observed to play different roles during flatfish development and metamorphosis (Lan-Chow-Wing et al., 2014). In mudskippers (*Boleophthalmus pectinirostris*) (Hong et al., 2014), MTs are involved in ovarian development, acting through the HPG axis in synchronization with the semilunar spawning rhythm. To date, two or three types of melatonin receptors have also been identified in European sea bass (*Dicentrarchus labrax*) (Sauzet et al., 2008), carp (*Catla catla*) (Moniruzzaman and Maitra, 2012), zebrafish (*Danio rerio*) (Reppart et al., 1996), golden rabbitfish (*Siganus guttatus*) (Park et al., 2006), and orange-spotted grouper (*Epinephelus coioides*) (Chai et al., 2013), and diurnal and seasonal variations in expression have demonstrated that melatonin affects the reproductive cycle.

Turbot (*Scophthalmus maximus*) is one of the most economically important marine flatfish species in Europe and northern China (Zhao et al., 2018a, b), and gonadal development and maturation in this species may be strongly affected by annual variation in photoperiod (Imsland et al., 2013). In artificial reproduction of turbot, the condition of exposing light is often utilized to regulate the time of maturation and spawning. To elucidate the mechanism governing gonadal development and maturation under changing light periods and investigate exogenously applied melatonin in the HPG axis during the breeding season of turbot, this study characterized cDNAs encoding three melatonin receptors (*Mtnrs*) in turbot, named *Mtnr1*, *Mtnr2*, and *Mtnr1c*, evaluated the concentration and localization of their mRNAs in the brain and gonad, and quantitatively assessed their mRNA abundance patterns over the entire reproductive cycle. Furthermore, the possible physiological roles played by melatonin were identified during gonadal maturation. The results of this study may help to characterize the role played by melatonin and its receptors, and clarify that melatonin is predominantly involved in gonadal development and gamete maturation in turbot.

2 Materials and methods

2.1 *Fish and samples*

All adult turbots were obtained from Oriental Ocean Sci-Tech Co., Ltd. (Shandong Province, China). Turbots were 4-year post-hatching, and not first sexually mature fish. Turbots throughout the reproductive season in males and females were collected every month. Before the tissues were obtained, turbots were anesthetized with a 0.05% solution of MS-222 (Sigma–Aldrich). Various tissues, including the brain, eye, skin, muscle, gill, liver, heart, spleen, kidney, stomach, intestine, liver and gonad, at stage II of gonadal development were rapidly excised, frozen in liquid nitrogen, and stored at –80°C until RNA extraction. Gonads, brains and pituitary glands from male and female turbot at different gonadal development stages (II, III, IV, V, and VI) were collected to investigate the expression profiles of *Mtnrs*. For each stage, three turbots were used for duplicates.

2.2 *Cloning full-length Mtnr1, Mtnr2 and Mtnr3 cDNA sequences in turbot*

Total RNA was extracted from the brains of turbot at stage II of gonadal development using the Simple P Total RNA Isolation Kit (BioFlux, China). Following the manufacturer's instructions, first-strand cDNA was first synthesized using a transScript first-strand cDNA synthesis kit (Transgen, China) with an oligo primer. Subsequently, cDNA fragments of the three turbot *Mtnrs* gene were amplified with primers (forward and reverse primers) (Table 1) that were designed according to the highly conserved regions of other fish. PCR was performed using a PTC-100 thermal cycle (Bio-Rad, Hercules, USA) with a PCR amplification procedure of denaturation at 94°C for 5 min, 30 cycles of amplification at 94°C for 30 s, 58°C for 1 min, 72°C for 30 s, and an additional elongation step at 72°C for 10 min. Finally, to clone the full-length coding sequence, 5′-RACE and 3′-RACE were performed using a SMARTer RACE cDNA amplification kit (Clontech, USA). The 5′- and 3′- ends of *Mtnrs* were amplified using a PCR anchor primer paired with gene-specific primers (GSPs) (Table 1). The two-round PCR programs were all performed on a PTC-100 thermal cycle (Bio-Rad). The diluted first-round PCR products served as templates for nested PCR with corresponding primers of GSP-nest (Table 1).

2.3 *Phylogenetic analysis*

Homology searches of deduced turbot Mtnr1, Mtnr2 and Mtnr3 sequences were performed using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple alignments of predicted amino acid sequences were conducted by BioEdit with the ClustalW alignment tool. A phylogenetic tree was constructed with Mega7 software by the neighbor-joining method using bootstrap analysis of over 1 000 replicates.

2.4 *Quantitative real-time PCR (qPCR)*

First, total RNA was extracted from each sample at different stages. Next, reverse transcription was performed at RT with 1 mg of total RNA using a PrimeScript RT Reagent Kit (Takara Bio Inc., China). All primers for qPCR analysis are listed in Table 1 and were validated with a PCR program and agarose gel electrophoresis. qPCR was performed in an ABI7300 real-time PCR instrument (Applied Biosystems, USA) with a SYBR premix ex taq kit (Takara Bio Inc., China) using the standard curve method with *β-actin* as the reference gene. The PCR procedure was pro-

Table 1. List of primers used for molecular cloning of Mtnr cDNAs and qPCR

Primer name	Nucleotide sequence (5′-3′)	Purpose/Products
Mtnr1-F	AAATGGGTCTCACCTGAACAGC	fragment PCR (759 bp)
Mtnr1-R	AAACGGCGAAGAGCACAAA	
Mtnr2-F	ACTGCTACATCTGTCACTCG	fragment PCR (480 bp)
Mtnr2-R	AGTAGGCCATGAAGTAGCTG	
Mtnr1c-F	CACTTTCTTATCCCGCTTCT	fragment PCR (424 bp)
Mtnr1c-R	GGCTTACTCTTCAGTCCCTC	
5′- *Mtnr1*-GSP1	CCCAGCAAACGGCGAAGAGCACAAA	5′RACE-PCR
5′- *Mtnr1*-GSP2	GACCAGCAGGTTGCCCAGGATGTCG	5′RACE-PCR (nested)
3′- *Mtnr1*-GSP1	TCTGCCACAGCCTCAAATATGATAAAC	3′RACE-PCR
3′- *Mtnr1*-GSP2	GTTTGTGCTCTTCGCCGTTTGCT	3′RACE-PCR (nested)
5′- *Mtnr2*-GSP1	CTCTCCTCGGTCTTCACTTTACG	5′RACE-PCR
5′- *Mtnr2*-GSP2	AGGTCAGCGAACGCCAAACTCAC	5′RACE-PCR (nested)
3′- *Mtnr2*-GSP1	CGCTGACCTCGTGGTAGCCTTCT	3′RACE-PCR
3′- *Mtnr2*-GSP2	GAGCCCTCGCCTCCGACCAAGTG	3′RACE-PCR (nested)
5′- *Mtnr1c*-GSP1	AAGAGCGAGGAGGATCGTCTTGTAC	5′RACE-PCR
5′- *Mtnr1c*-GSP2	CCTCAGGCTGAACAAACGGTCGTAG	5′RACE-PCR (nested)
3′- *Mtnr1c*-GSP1	GAAAGTGGCGCCCCACATTCCTGAGTG	3′RACE-PCR
3′- *Mtnr1c*-GSP2	GTAGCGGAGATGAATGTATGAACAACTG	3′RACE-PCR (nested)
sm- *Mtnr1*-F	AACCTGGGTTACGTCCACTG	224 bp
sm- *Mtnr1*-R	AGCGAACCCACAAAGAGGTT	
sm- *Mtnr2*-F	CGTGGTCTTTGTGCTGTTCG	204 bp
sm- *Mtnr2*-R	ATGCGCTTGTACTCGTTCCT	
sm- *Mtnr1c*-F	CAAGACGATCCTCCTCGCTC	185 bp
sm- *Mtnr1c*-R	GGCGAGGCTCCAGATAACAA	
sm-*β-actin*-F	GCTGTGCTGTCCCTGTATGCC	187 bp
sm-*β-actin*-R	AGGAGTAGCCACGCTCTGTCA	

grammed according to the manufacturer's protocol. A dissociation curve was added at the end of each program to determine the amplification specificity.

2.5 *Measurement of serum melatonin, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels*

Before taking tissues from turbot, approximately 10 mL of blood was collected from the caudal vein. Next, the blood samples were incubated at 4°C overnight and centrifuged at 3 000 r/min for 10 min at 4°C. The serum was removed from the supernatant and stored at –80°C for analysis. Levels of melatonin, LH and FSH were measured by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay. Melatonin ELISA kits (IBL, Germany) and LH and FSH ELISA kits (Bnibt, China) were utilized. The test experiment was performed by Union Medical and Pharmaceutical Technology (China).

2.6 *Statistical analysis*

All the data were expressed as the mean ± SEM. Statistics were performed using SPSS 15.0 software (www.ibm.com/software/analytics/spss). The changes in gene expression during gonadal stages were analyzed by one-way ANOVA followed by the Bonferroni multiple comparison test. The value of p less than 0.05 were considered to be significant, and different letters in the figures demonstrate significant differences between gonadal stages.

3 Results

3.1 *Cloning and phylogenetic analysis of Mtnr cDNAs*

First, three *Mtnr* complete coding sequences were successfully obtained using 3′- and 5′-RACE in turbot. The full-length turbot *smMtnr1*, *smMtnr2*, and *smMtnr1c* cDNAs (GenBank accession Nos MK738109, MK738110 and MK738111) were 1 360 bp, 1 667 bp and 1 773 bp in length, respectively. The three cDNAs contained open reading frames of 1 071 bp, 1 140 bp and 1 086 bp encoding precursor proteins of 357, 379 and 362 deduced amino acids (aa), respectively (Fig. 1).

All three Mtnr-deduced proteins contained seven transmembrane region motif profiles. The alignment analysis the deduced amino acid sequences of turbot Mtnr1, Mtnr2, and Mtnr1c with other species is shown in Fig. 2. The characteristics of three Mtnrs of turbot listed in Table 2.

All the fish sequences of the alignment analysis of each Mtnr showed high homology. Meanwhile, turbot Mtnr1 showed 95% identity and 96% similarity with Mtnr1 from *Paralichthys olivaceus*, 94% identity and 95% similarity with Mtnr1 from *Cynoglossus semilaevis*. Notably, turbot Mtnr2 showed the highest identity (95%) and similarity (96%) with Mtnr2 of *Lates calcarifer*. Compared to *Lates calcarifer*, the identity and similarity with *Paralichthys olivaceus* were 94% and 95%, and *Cynoglossus semilaevis* 92% and 94%, respectively. Turbot Mtnr1c also shared high identity and similarity with other fish species (88%–97%). More specifically, turbot Mtnr1c was 97% identical to Mtnr1c from *Larimichthys crocea* and 93% identical to *Paralichthys olivaceus*, and it exhibited high similarity with Mtnr1c from *Larimichthys crocea* (97%) and *Solea senegalensis* (97%). Phylogenetic analysis indicated that the three turbot Mtnrs and those from other typical vertebrates clustered into three separate branches: the MT1 branch, MT2 branch and MT3 branch (Fig. 3). Each turbot Mtnr is closely related to the teleost separately.

3.2 *Expression of turbot Mtnrs in different tissues*

The specific expression of turbot tissues in the gonadal development of stage II was investigated by qPCR. All the samples

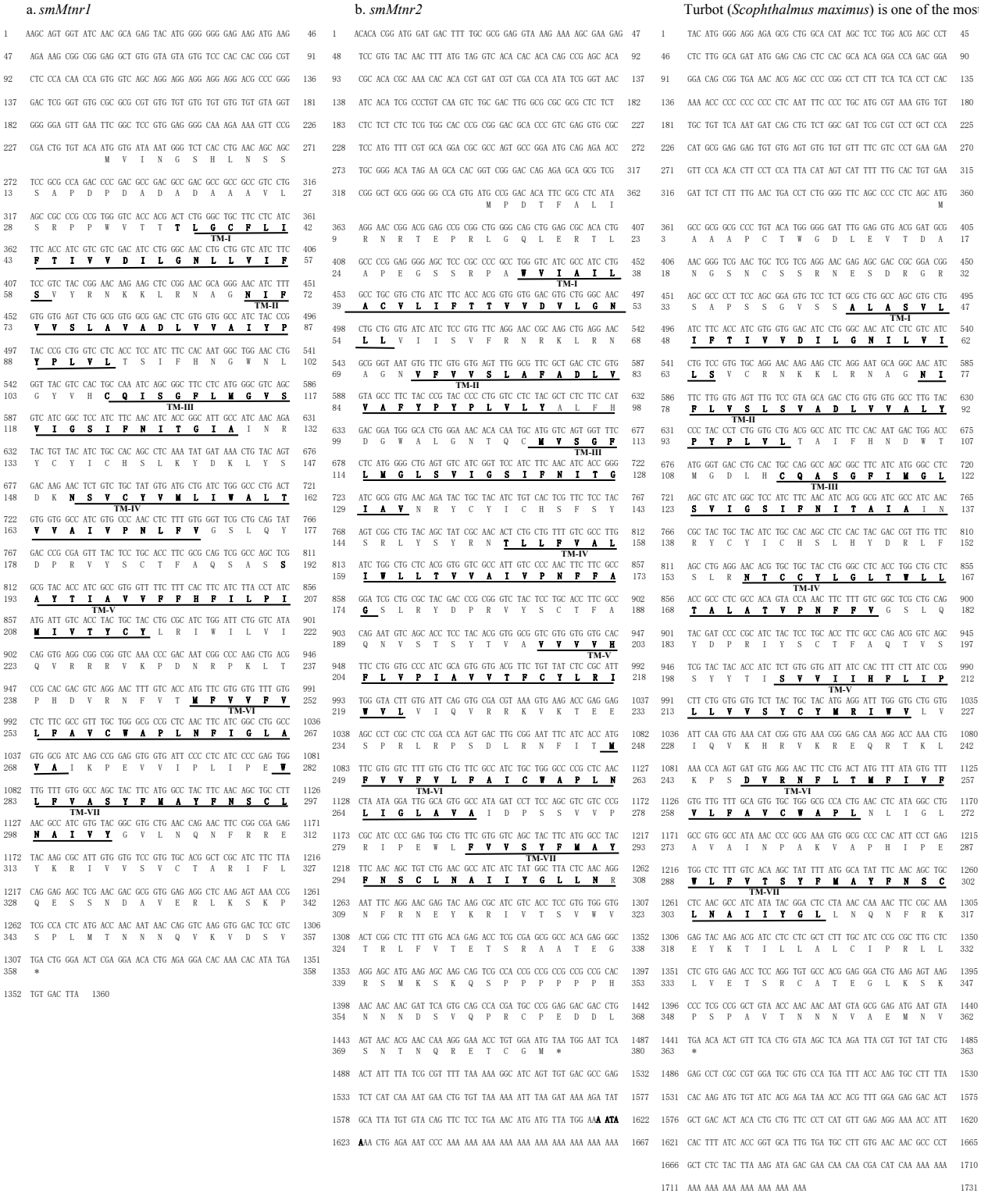
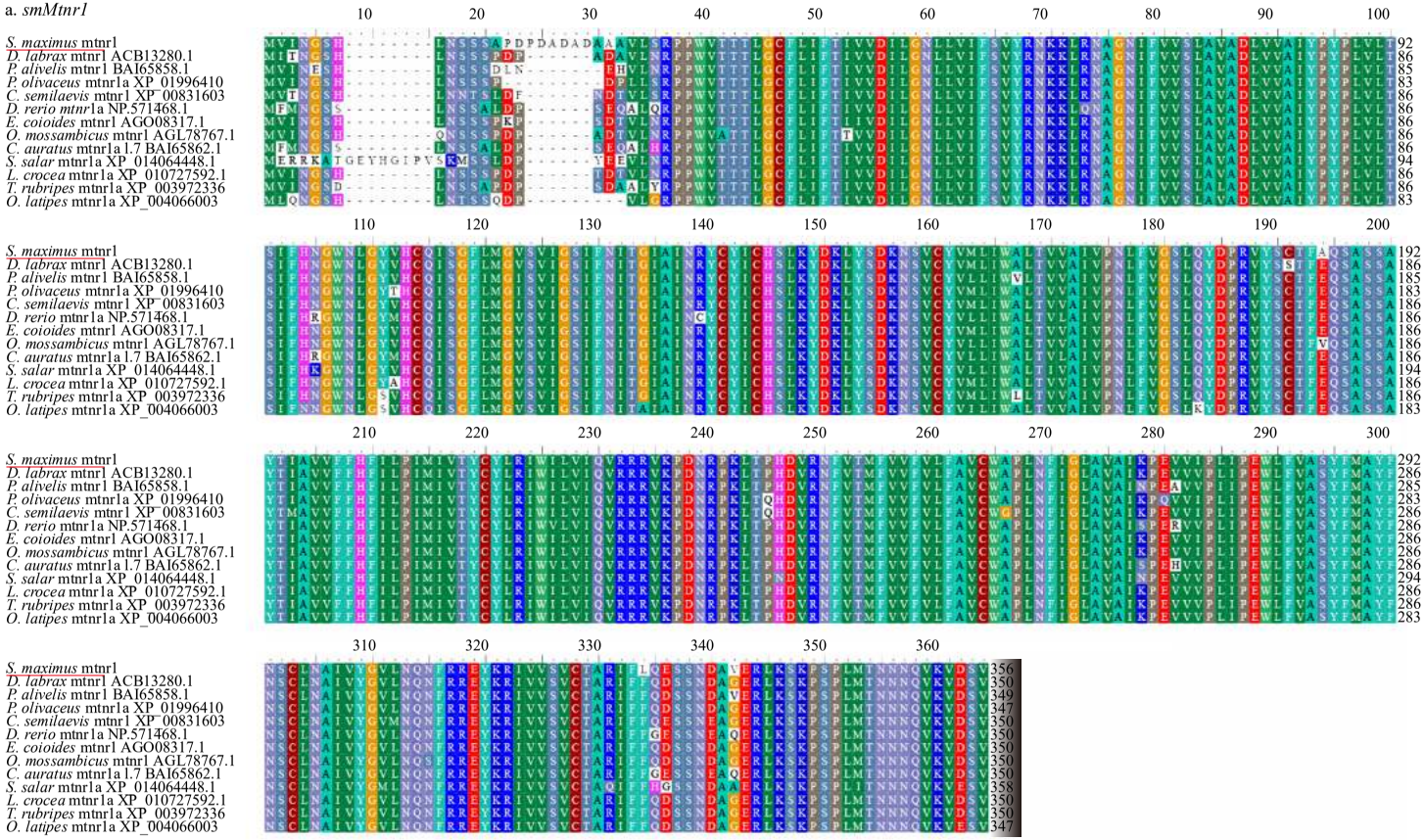


Fig. 1. The full-length cDNA and deduced amino acid sequence of *smMtnr1* (a), *smMtnr2* (b), and *smMtnr1c* (c). The transmembrane region is underlined. TM I-VII: transmembrance domain.

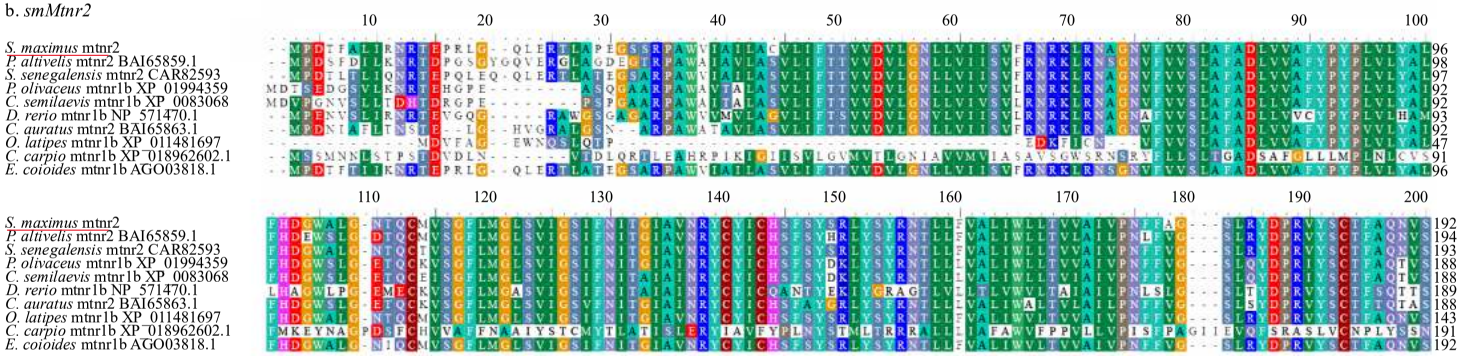
were collected at night from male turbot. Three *Mtnr* mRNAs were all highly expressed in the whole brain (Fig. 4). The expression of *smMtnr1* and *smMtnr2* was considerably higher than that of *smMtnr1c*. Meanwhile, *smMtnr1* and *smMtnr2* mRNAs were also concentrated in the gonad and eye. However, *smMtnr1c* mRNA was also expressed in some other tissues. Specifically, *smMtnr1c* mRNA was expressed relatively strongly in skin with weak expression in the eye and liver followed by the brain, muscle and gonad.

The expression patterns of three *smMtnrs* mRNAs in central brain areas were also analyzed in this study (Fig. 5). According to the microscopic imaging of brain sections, fresh turbot brain was divided by scalpel into seven areas: the olfactory bulbs (Ob), the telencephalon (Te), including the telencephalon and preoptic

a. *smMtnr1*



b. *smMtnr2*



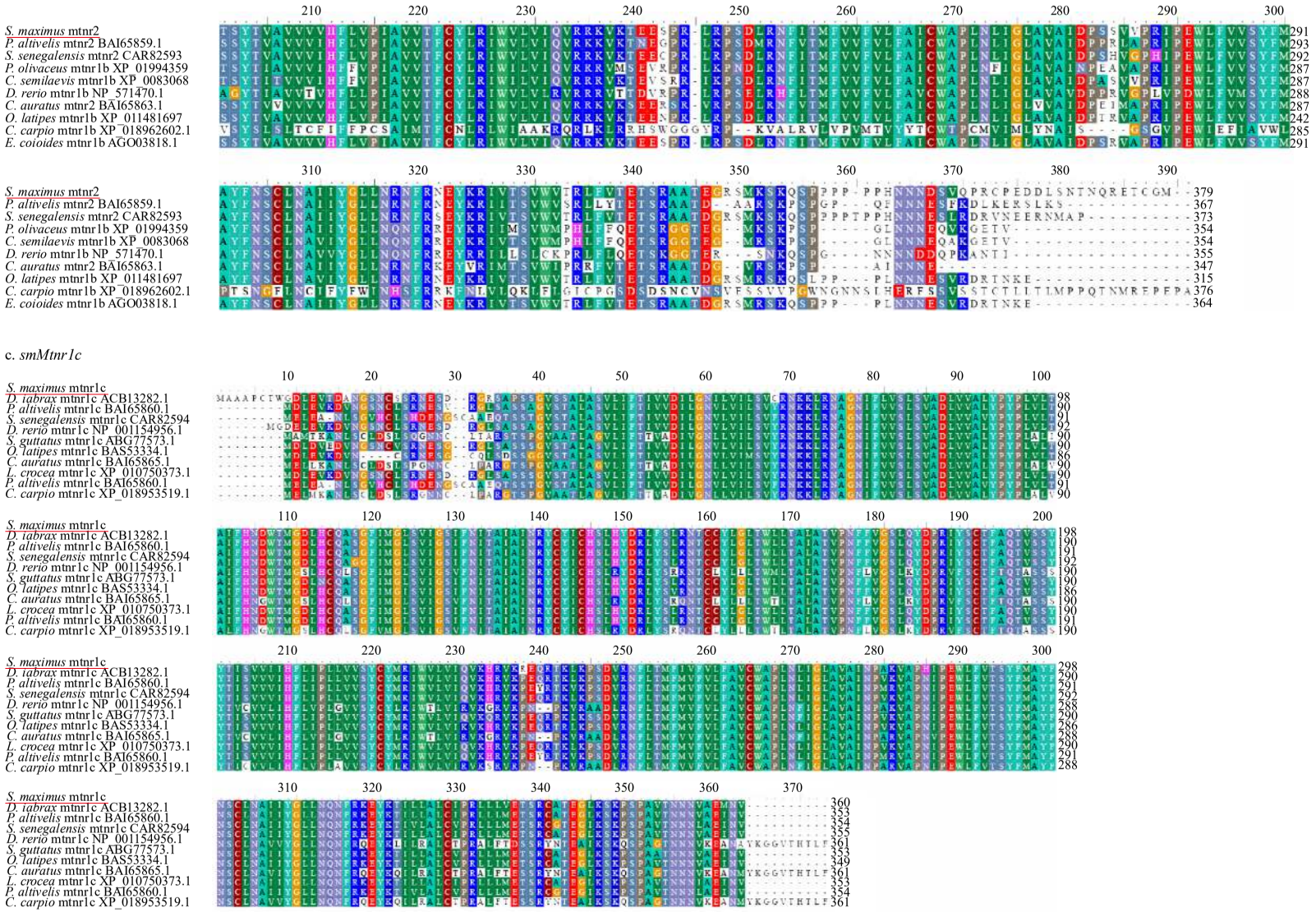


Fig. 2. Multiple alignment of the deduced amino acid sequences of *smMtnr1* (a), *smMtnr2* (b), and *smMtnr1c* (c) from turbot and several other different animals. Conservative and unrelated residues are shaded in different color, respectively. Abbreviation: *Carassius auratus* (*C. auratus*), *Cyprinus carpio* (*C. carpio*), *Cynoglossus semilaevis* (*C. semilaevis*), *Dicentrarchus labrax* (*D. labrax*), *Danio rerio* (*D. rerio*), *Epinephelus coioides* (*E. coioides*), *Oryzias latipes* (*O. latipes*), *Larimichthys crocea* (*L. crocea*), *Oreochromis mossambicus* (*O. mossambicus*), *Plecoglossus altivelis* (*P. altivelis*), *Paralichthys olivaceus* (*P. olivaceus*), *Siganus guttatus* (*S. guttatus*), *Schophthalmus maximus* (*S. maximus*), *Salmo salar* (*S. salar*), *Solea senegalensis* (*S. senegalensis*), *Takifugu rubripes* (*T. rubripes*).

Table 2. Characteristics of *Mtnrs* in turbot

Gene	Chr	CDS	pI	MWs	Transmembrane	Transmembrane regions
Mtnr1	8	357	8.90	40 001.32	7 TM	36–58, 70–92, 106–129, 149–172, 193–214, 247–269, 282–302
Mtnr2	4	379	9.32	42 846.41	7 TM	33–55, 72–98, 109–131, 152–174, 199–221, 248–270, 285–308
Mtnr1c	13	362	8.65	40 211.51	7 TM	42–64, 76–98, 113–137, 155–178, 203–225, 246–267, 288–310

Note: CDS, coding sequence; Chr, chromosome; pI, isoelectric point; MWs, molecular weights.

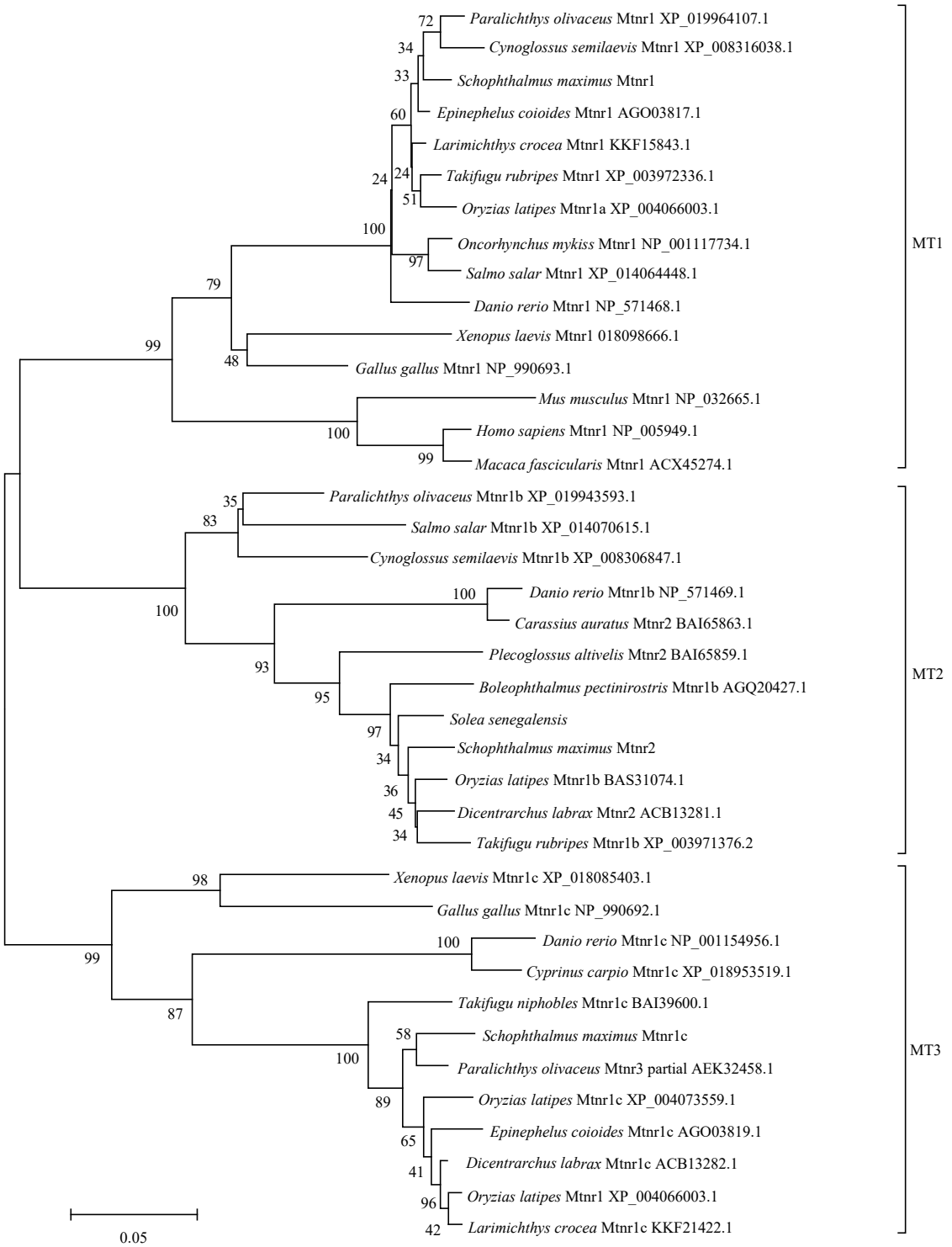


Fig. 3. Phylogenetic tree of deduced amino acid sequences for three *smMtnrs* from turbot and other vertebrates was constructed by Mega7 with neighbor-joining method. The numbers adjacent to nodes indicate bootstrap percentage value for 1 000 replicates (>80%). The GenBank accession numbers of the sequences presented before the species name; and *smMtnr1*, *smMtnr2*, and *smMtnr1c* cDNAs are with the GenBank accession numbers of MK738109, MK738110 and MK738111.

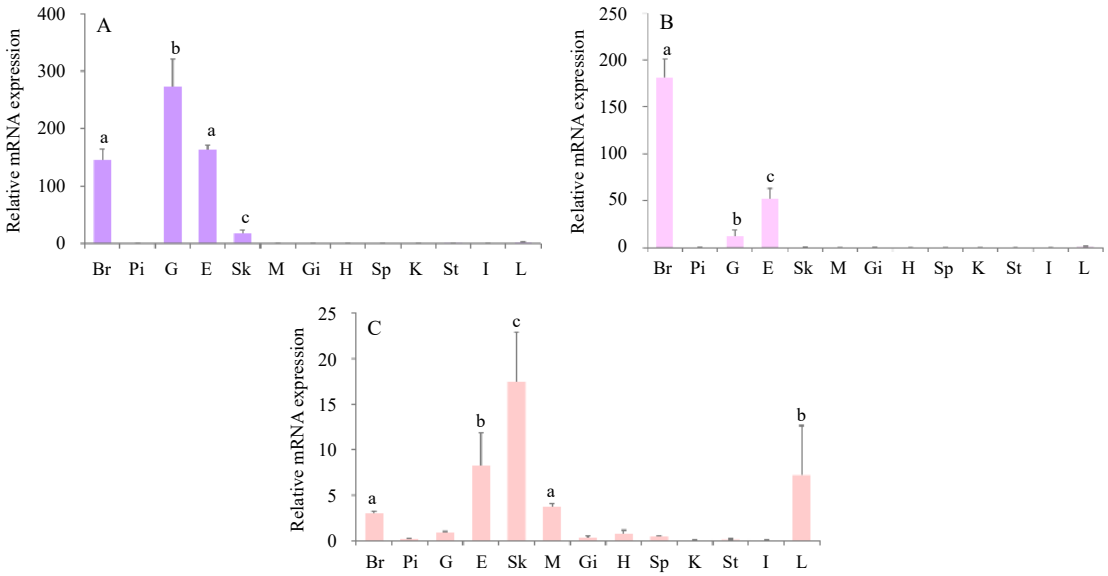


Fig. 4. The tissue distribution of three *smMtnr* genes in turbot. A. Expression of *smMtnr1*; B. expression of *smMtnr2*; C. expression of *smMtnr1c*. The relative abundance of three *smMtnr* genes were expressed as mean ± SEM (n=3). Tissue abbreviations: Br, brain; Pi, pituitary; G, gonad; E, eye; Sk, skin; M, muscle; Gi, gill; H, heart; Sp, spleen; K, kidney; St, stomach; I, intestine; L, liver. Different letters above bars represent statistical significant ($p < 0.05$).

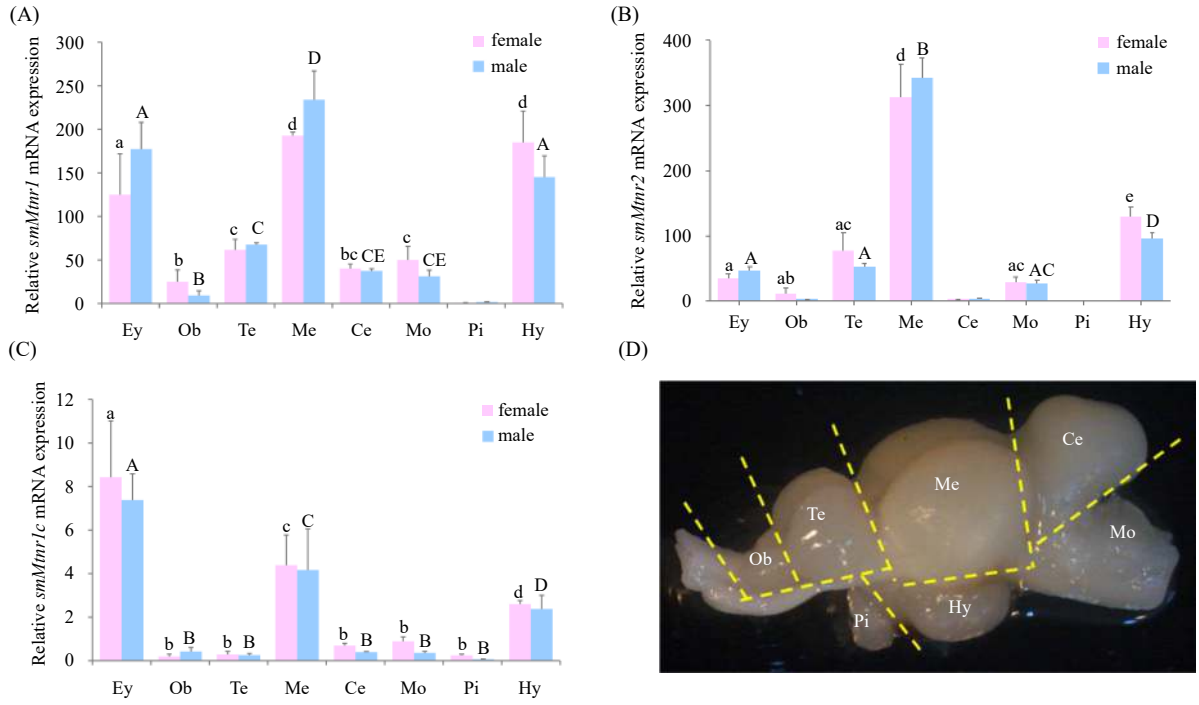


Fig. 5. Expression of three *smMtnr* genes in different brain areas and eye. (A) Expression of *smMtnr1*; (B) expression of *smMtnr2*; (C) expression of *smMtnr1c*; (D) dissected brain areas used for expression studies. Ey, eye; Ob, olfactory bulbs; Te, telencephalon; Me, mesencephalon; Ce, cerebellum; Hy, hypothalamus; Pi, pituitary gland; Mo, medulla oblongata. Different letters above bars represent statistical significant ($p < 0.05$).

area (POA), the mesencephalon (Me), including the mesencephalon and thalamus, the hypothalamus (Hy), including the hypothalamus and saccus vasculosus, the cerebellum (Ce), the medulla oblongata (Mo) and the pituitary gland (Pi). The expression of *smMtnrs* mRNAs in the eye was also compared with that in the central brain. Overall, the three *smMtnr* mRNAs were detected in all analyzed brain areas and eyes and exhibited similar expression patterns. Specifically, the expression was evident in Me, Hy and the eye. Next, there was a decrease in the Te, Mo, Ob and Ce. However, all the *Mtnr* mRNAs were weakly detected in Pi. In addition, the expression patterns of *Mtnrs* mRNAs in the female turbot brain were similar to those in the male turbot brain.

3.3 *Expression profiles of smMtnrs mRNAs in brain and gonad during gonadal development stages*

Throughout the reproductive stage, gonadal development in

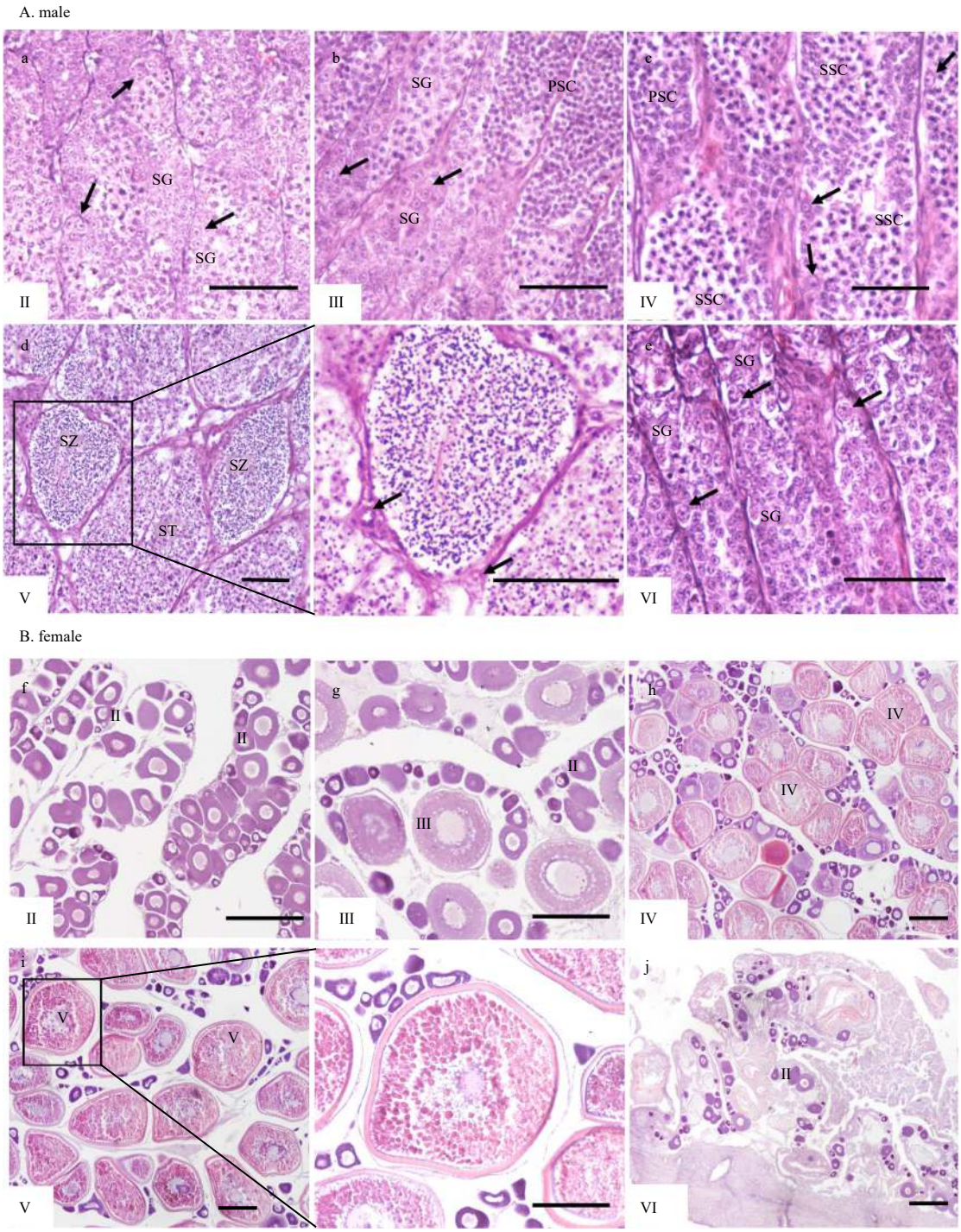


Fig. 6. Histology of testes and ovaries during reproductive cycle. Tissue abbreviations: PSC, primary spermatocytes; SG, spermatogonia; SSC, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; I, previtellogenic oocytes; II, primary vitellogenic oocytes; III–IV, large growth of vitellogenic oocytes. Scale: A (a–e), 50 μm; B (f–j), 200 μm.

males and females could be divided into five stages. For male turbot (Fig. 6A), the five gonad stages were stage II (mostly spermatogonia), stage III (primary spermatocytes together with spermatogonia), stage IV (secondary spermatocytes together with spermatogonia), stage V (spermatids or spermatozoa together with spermatogonia), and stage VI (mostly spermatogonia). For female turbot (Fig. 6B), the five gonad stages were stage II (primary growth oocytes), stage III (early vitellogenesis), stage IV (late vitellogenesis), stage V (ovulated oocytes), and stage VI (postovulatory follicle in degenerated ovaries).

First, the mRNA expression of the three *smMtnr* genes in the whole brain in each gonadal development stage is shown in Fig. 7. In general, in male and female turbot, from stages II to VI, brain *smMtnr* mRNA expression had a similar tendency, gradually decreasing and reaching the lowest levels at the V stage and subsequently increasing at the IV stage. However, the mRNA expression level of *smMtnr1c* in the female turbot brain was not significantly different. Next, the mRNA expression levels of the three

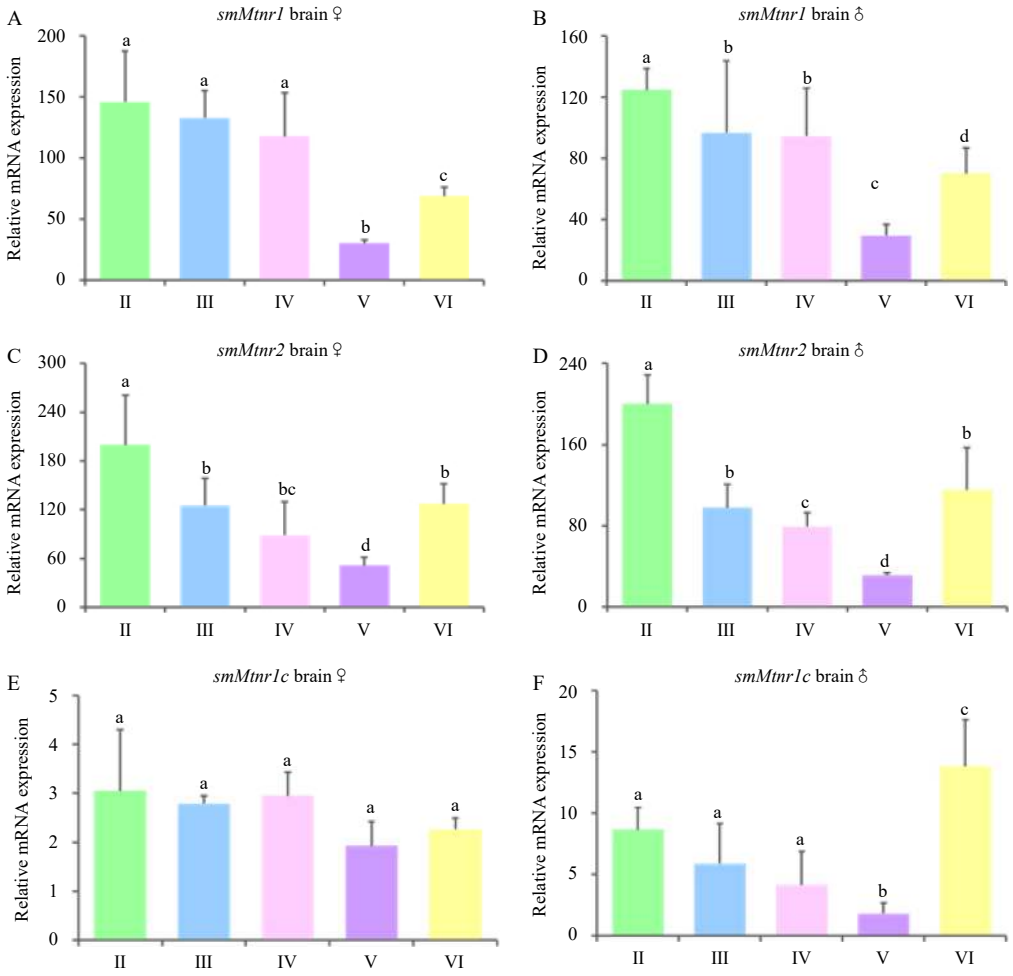


Fig. 7. Expression of three *smMtnr* genes throughout the reproductive cycle in turbot brain of male and female. Error bars are presented as the mean±SEM. Different letters above bars represent statistical significance ($p < 0.05$) between two sexual maturation stages. The internal control was *β-actin*.

smMtnr in gonads at each developmental stage were also investigated (Fig. 8). Notably, the expression levels of *smMtnr* mRNAs in male and female gonads were quite different. In the ovary, *smMtnr1* mRNA gradually increased from stages II to IV, reached its highest level at stage IV, and later decreased at stages V to VI. Although the levels of *smMtnr2* mRNA and *smMtnr1c* mRNA shared a similar tendency with *smMtnr1*, their highest expression level occurred at stage V. In the testes, the mRNA expression of all three smMtnrs decreased gradually and reached the lowest level at mature testes at stage V.

Meanwhile, based on *smMtnr* mRNA expression in different brain areas, their expression levels in the Me and Hy in the immature stage, breeding season and postbreeding season were also investigated (Fig. 9). For *smMtnr1* mRNAs and *smMtnr2* mRNAs in Me and Hy of males and females, the expression levels at the breeding season were lower than those at the immature stage and postbreeding season. For *smMtnr1c* mRNAs, the expression in Me of males and Hy of females during the breeding season was slightly higher than that in the immature stage and postbreeding season. However, *Mtnr1c* mRNA expression showed a rising trend from the immature stage to the postbreeding season. In general, the mRNA expression of *smMtnr1* and *smMtnr2* was considerably higher than that of *smMtnr1c*.

3.4 *Serum melatonin and LH, FSH concentration*

The serum melatonin concentration exhibited a variation trend with the reproductive stage development of males and females (Fig. 10). The melatonin profiles gradually increased from stages II to V and reached the highest level at stage V followed by a decrease at stage VI. Showing a similar pattern to serum melatonin, the serum LH concentration significantly increased from stages II to V and decreased at stage VI. However, FSH concentrations in females and males were notably different. In females, the FSH concentrations at stages V and VI were considerably lower than those at stages II to IV. In males, the lowest concentrations occurred at stages IV and V.

4 Discussion

In the present study, we elucidated the molecular basis of the melatonin receptor system in turbot by characterizing the three *Mtnrs* genes (*smMtnr1*, *smMtnr2*, and *smMtnr1c*) and analyzing *Mtnrs* genes and melatonin concentrations in the brain and gonad during reproductive development. All three Mtnr deduced proteins contained seven transmembrane region motif profiles, which are characteristic of the G-protein-coupled receptor family (Confente et al., 2010; Park et al., 2007a). Meanwhile, the identified Mtnrs belong to three high affinity receptor subtypes. The Mtnr1 and Mtnr2 subtypes existed in all vertebrates, and the Mt-

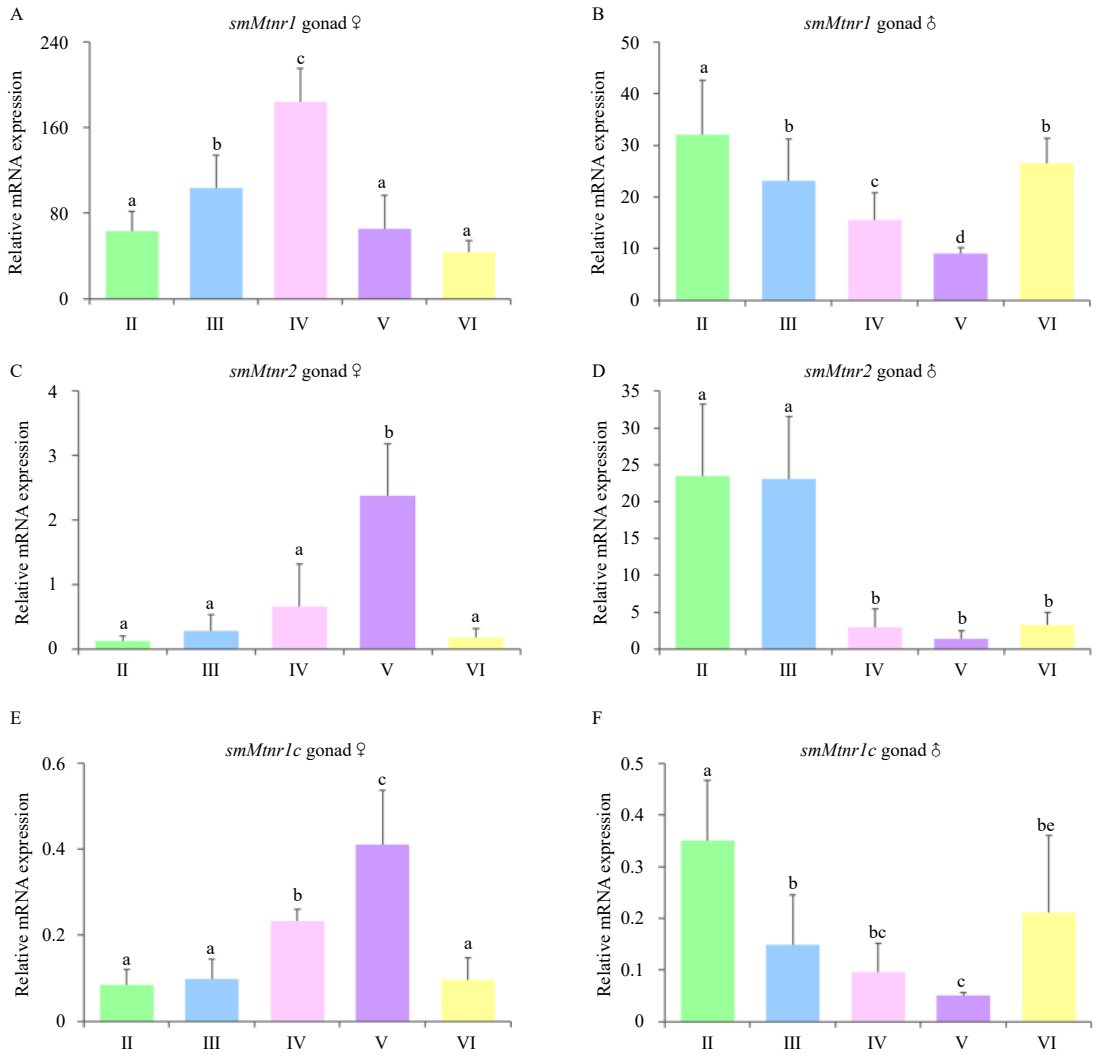


Fig. 8. Expression of three *smMtnr* genes throughout the reproductive cycle in turbot gonad of male and female. Error bars are presented as the mean±SEM. Different letters above bars represent statistical significance ($p < 0.05$) between two sexual maturation stages. The internal control was *β-actin*.

nr1c subtype is found only in nonmammalian vertebrates (Nishiwaki-Ohkawa and Yoshimura, 2016; Sauzet et al., 2008). Notably, in teleosts, there are two different MT1 receptor subtypes, Mtnr1a 1.4 and Mtnr1a 1.7, in such species as zebrafish, rainbow trout, goldfish, Senegalese sole, and olive flounder (David et al., 1999; Ikegami et al., 2009; Lan-Chow-Wing et al., 2014; Reppart et al., 1996; Shin et al., 2011). Based on the molecular characteristics and distinctive branch representation, *smMtnr1* in turbot appears to belong to the Mtnr1a 1.4 subgroup.

Tissue expression patterns showed that *smMtnrs* were highly expressed in the brain, gonad and eye, in general agreement with the results from previous studies (Hong et al., 2014; Sauzet et al., 2008). It is found that some differences exhibit in the *Mtnrs* expression levels in fish gonad. The *smMtnr1* mRNA highly expressed, and *smMtnr2* lower, *smMtnr1c* lowest in the gonad. Melatonin receptors could be detected in testis of European sea bass (*Mtnr2*) (Sauzet et al., 2008), in ovary of goldfish (*Mel1a 1.7* and *Mel1b*) (Ikegami et al., 2009), in testis of Atlantic salmon (mtnr1aaα, mtnr1ab, mtnr1b) (Ciani et al., 2019). It was also reported that the *Mtnr1* and *Mtnr2* mRNA expressed in ovarian tissues (Moniruzzaman and Maitra, 2012). The differences may account for species related. These results suggested the melatonin receptors, especially of *smMtnr1* might directly act at gonad level. It is worth noting that there is no *Mtnr1c* expression in gonad of mudskipperin. However, it is detectable in turbot. Further investigations are necessary to clarify the Mtnr1c function in turbot gonad. It is interesting that, *smMtnr1* also expressed in gill, consistent with the research of sea bass, suggesting that the gill as a richly vascularized organ, is probably tissue specific expression (Sauzet et al., 2008). We also found that, *smMtnr1c* was highly expressed in the skin, to a much lower levels of expression in gonad and brain, different with sea bass and golden rabbitfish. The reasons might be different reproductive status or daytime when the experiments were done (Park et al., 2007b).

In terms of *Mtnrs* expression in turbot, all the three *smMtnrs* was observed in central brain regions, including Me, Hy and five other parts. The expression levels of *Mtnr1* and *Mtnr2* appeared to be higher than that of *smMtnr1c*. In the previous study, in Senegalese sole (Confente et al., 2010), golden rabbitfish (Park et al., 2006, 2007b), European sea bass (Sauzet et al., 2008), and mudskipper (Hong et al., 2014), the expression of *Mtnr1* and *Mtnr2* decreased gradually with gonadal maturation. Moreover, *Mtnr1* mRNAs were also observed to be abundant in the retinal pigment epithelium (Sauzet et al., 2008). However, the expression of

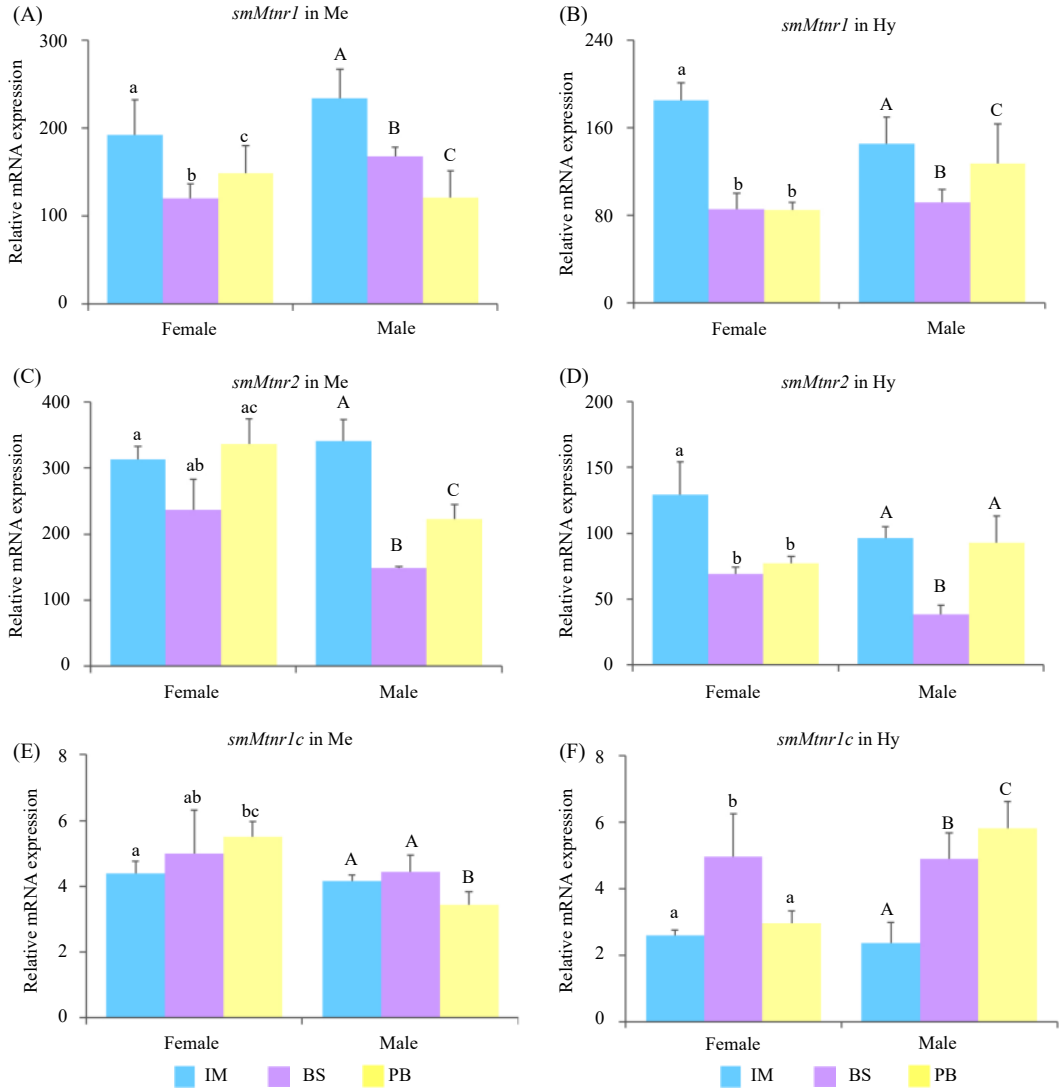


Fig. 9. Expression of three *smMtnr* genes in mesencephalon (Me) and hypothalamus (Hy) in immature stage (IM), the breeding season (BS) and post-breeding season (PB). Error bars are presented as the mean±SEM. Different letters above bars represent statistical significance ($p < 0.05$). The internal control was *β-actin*.

smMtnr1c in the central nervous system (CNS) was notably low in turbot. Indeed, in previous research, *Mtnr1c* expression exhibited wide variability in fish and birds with a species-specific expression pattern (Ikegami et al., 2009), and there was no regularity in the expression of *Mtnr1c*. Taken the findings of previous investigations together, it is suspected that Mtnr1 and Mtnr2 may play vital roles in the gonadal development of turbot.

In the seasonal reproduction of turbot farms, when the temperature was maintained at 13°C to 15°C, a progressively longer daylength was generally utilized to treat the brood stock to stimulate spermatogenesis, oogenesis and maturation of the gonads, indicating that changing the daylength shifted the spawning time (Alvariño et al., 2001; Forés et al., 1990; Peleteiro-Alonso et al., 1995). Melatonin is produced at the pineal organ and the retina, and is known as the key signal of photoperiodic information to the CNS in vertebrates (Carnevali et al., 2010). Previous studies have confirmed that melatonin synthesis and secretion during scotophase respond to external photoperiod changes in fishes, and that fluctuations in melatonin secretion affect the reproductive endocrine system (Coon and Klein, 2006; Kim et al., 2020; Zhu et al., 2014). What is the mechanism of melatonin on functions of gonads in season breeding? Controlling of processes of seasonal rhythms is widely accepted. Melatonin implants on the turbot reproductive system showed supraphysiological plasma melatonin concentrations, which were approximately one hundred times higher in females, and gonadal recrudescence started 45 d earlier, with a higher spermatozoa concentration being observed in males, three months after implantation (Alvariño et al., 2001). Another strategy lays in the high affinity melatonin receptors. In this study, with increasing light, *Mtnr1* and *Mtnr2* expression levels decreased with gonadal development, reached their lowest levels at the maturation stage, and increased significantly in the degeneration stage (after light recovery). The melatonin profiles gradually increased from stages II to V and reached the highest level at stage V. The results mainly indicated that melatonin functioned synchronously through their receptors and as hormone or antioxidant in turbot reproduction (Maitra et al., 2016).

Melatonin implants induced the expression of FSH and LH and a trend towards higher sex steroid hormones in the eel (*Anguilla anguilla*) (Sébert et al., 2008). Indeed, many studies high-

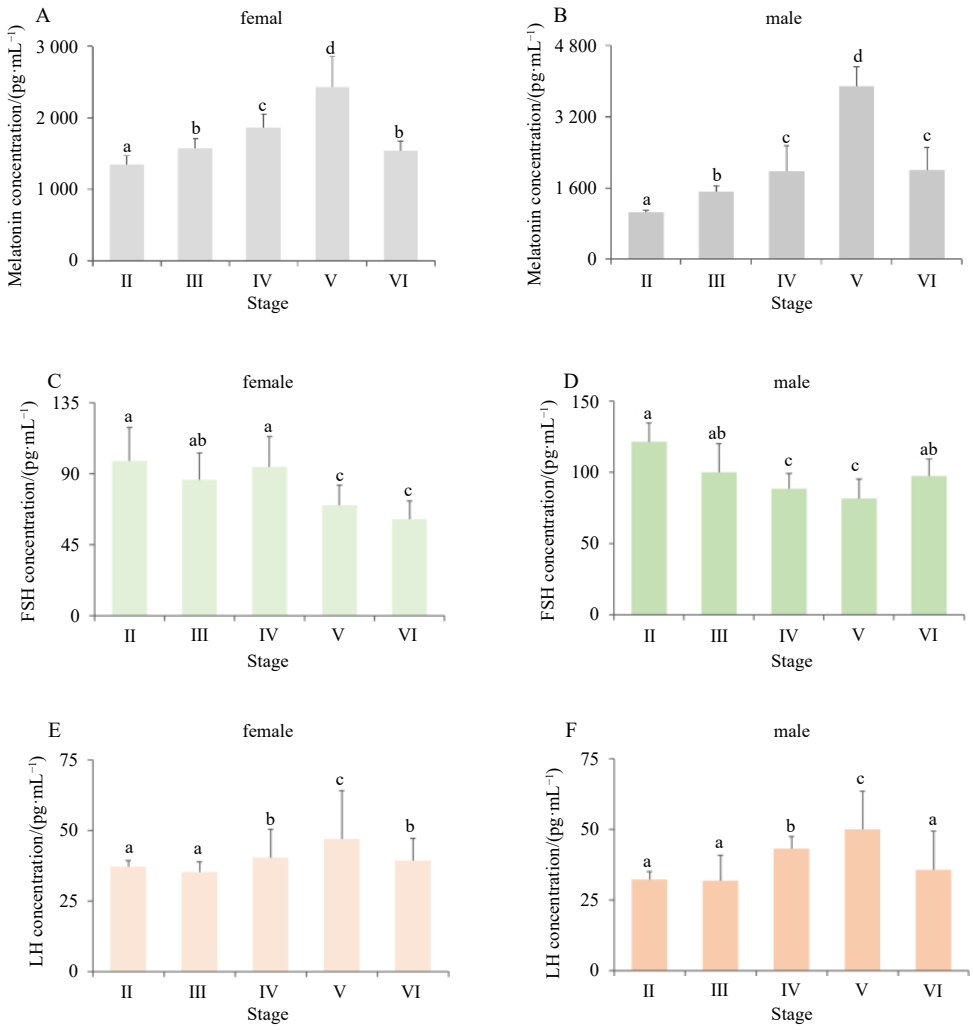


Fig. 10. Serum levels of melatonin, FSH and LH throughout the reproductive cycle in turbot of male and female. Different letters above bars represent statistical significance (p<0.05).

lighted the environmental regulation of daily and annual melatonin variations, such as light, temperature and salinity, as well as self-sustained endogenous rhythms, and melatonin is known as a potent candidate in the regulation of fish oocyte growth and maturation (Maitra et al., 2013). Considering these findings, it is suggested that melatonin plays an important role in photoperiodic signal transduction and regulates annual reproduction and gonadal development in turbot. In addition, *in vitro* studies are essential to clarify that melatonin affects the mechanism of brood stock maturation, the on-off action of melatonin and its receptor on the HPG axis in fish. Some studies had done to understand the mechanisms by which melatonin and its receptor act on the HPG axis. It seems likely that melatonin receptors expression in the pituitary glands influences the reproductive cycle. Gonadotropin-releasing hormone (GnRH), which is synthesized and released by hypothalamic neurosecretory cells (Kochman, 2012), possibly interacts between melatoninergic and GnRH systems (Maitra and Hasan, 2016). Meanwhile, melatonin also could act on gonadotropin-inhibitory hormone (GnIH) neurons to stimulate GnIH (Kazuyoshi et al., 2015). GnRH and GnIH represent a substrate of photoperiod effects on reproduction. The new hypothalamic peptides—kisspeptins (KISS) has been confirmed to control GnRH secretion and regulates gonad development (Gopurappilly et al., 2013; Thommai et al., 2015). Thus, melatonin might involve in fish seasonal reproduction through the interplay between the GnRH, GnIH and KISS on the HPG axis (Falcón et al., 2007; Ikegami and Yoshimura, 2016). What's more, melatonin receptors also are presented in carp oocytes and exhibit in each reproductive phase with the reproduction (Maitra et al., 2013; Moniruzzaman et al., 2016). But, to clarify the mechanism by which melatonin regulates fish reproduction remains more future research.

Current studies have shown that melatonin also has lipophilic characteristics and can act directly as a scavenger of free radicals because it easily crosses the plasma membrane of cells (Moniruzzaman et al., 2016; Rodriguez et al., 2004; Tan et al., 1993). During oogenesis, especially in the stage of oocyte maturation and ovulation, a large amount of free radicals is generated and causes elevated oxidative stress (Tamura et al., 2008). In mammals and teleosts, many studies have demonstrated that melatonin can scavenge free radicals and stimulate antioxidative enzymes, indicating a new physiological role for melatonin as a potent antioxidant (Gaeta et al., 2002; Galano et al., 2013; Rodriguez et al., 2004; Zhang and Zhang, 2014). On the other hand, melatonin receptors have been found in the ovaries of mammals and fishes (Chattoraj et al., 2009; Soares et al., 2003). Melatonin also directly acts on the ovary. Therefore, melatonin not only plays the role of a hormone but is also associated with a reduc-

tion in oxidative stress to induce final oocyte maturation and spawning (Maitra and Hasan, 2016). The *Mtnrs* and melatonin in turbot were detected at high levels during the reproductive period. The results emphasized that turbot melatonin may coordinate its seasonal breeding and regulate oocyte functions. However, more research should be performed to detect the influence of melatonin on turbot preovulatory follicles under oxidative stress and to verify its function in improving the quality of turbot oocytes.

Melatonin and the pineal gland also play a crucial role in early development by accelerating the cell proliferation of fish (Danilova et al., 2004; Ziv and Gothilf, 2006). Indeed, the pineal gland develops earlier than the retina in structure and function. Specifically, the development of photoreceptor cells, light pigment molecules and melatonin biosynthesis in the pineal gland occurs prior to retina development (Vuilleumier et al., 2006). Some studies have investigated whether melatonin regulates light conduction during embryo incubation (Forsell et al., 1997). In flatfish, several studies have highlighted that thyroid hormones drive morphological, molecular and physiological changes during metamorphosis (Power et al., 2001). However, melatonin, acting as an antagonist of thyroid hormones, could also be an important component of the hormone signaling pathway to shift metamorphosis in anuran amphibians (Wright, 2002). In addition, research in Senegalese sole showed that melatonin receptors were strongly expressed during early development and metamorphosis, suggesting different roles in flatfish development and metamorphosis (Lan-Chow-Wing et al., 2014). Thus, as a typical flatfish, turbot melatonin may be involved in the biological processes of early metamorphosis.

In conclusion, this study investigates three *Mtnr* in turbot, *Mtnr1*, *Mtnr2*, and *Mtnr1c*, adding complementary *Mtnr* to fish. The *Mtnr* mRNA abundance patterns in the brain and gonad were quantitatively assessed during the entire reproductive cycle. Furthermore, the concentrations of melatonin, as well as those of FSH and LH, were identified. The results suggested that melatonin in the nervous system is probably the main factor regulating the physiological functions of turbot seasonal reproduction. Moreover, the results of this study may facilitate further efforts to identify fish melatonin receptors involved in gonadal development and gamete maturation.

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