fishes

MDPI

Article

Functional Involvement of Melatonin and Its Receptors in Reproductive Regulation of the Marine Teleost, Large Yellow Croaker (*Larimichthys crocea*)

Xudong Liang †, Jixiu Wang †, Baoyi Huang, Haojie Yuan, Yucheng Ren, Chenqian Wu, Tianming Wang * and Jingwen Yang *

National Engineering Laboratory of Marine Germplasm Resources Exploration and Utilization, Marine Science and Technology College, Zhejiang Ocean University, Zhoushan 316022, China
* Correspondence: wangtianming@zjou.edu.cn (T.W.); yangjingwen@zjou.edu.cn (J.Y.)
† These authors contributed equally to this work.

Abstract: Melatonin is a critical regulator of biological rhythms across organisms, transducing light signals into neuroendocrine signals that facilitate reproductive regulation in response to environmental cues. However, the precise mechanisms through which melatonin regulates reproduction in fish require further investigation. In this study, we employed molecular and organizational biological techniques to examine the expression patterns of melatonin and its five receptor subtypes (*LcMTNR1A1*, *LcMTNR1A2*, *LcMTNR1B1*, *LcMTNR1B2*, and *LcMTNR1C*) in various tissues of the large yellow croaker (*Larimichthys crocea*). Our results revealed significant expression of all receptors in the pituitary and testes, with distinct gender differences, including a lack of expression in the ovary. Moreover, our data indicate that melatonin and its receptors are primarily expressed during stage III, highlighting their role in sexual maturity. Enzyme- linked immunosorbent assay (ELISA) results further demonstrated that in vitro melatonin incubation in the brain of *L. crocea* influenced gonadotropin-releasing hormone (GnRH) and testosterone secretion in a dose-dependent manner, suggesting actions beyond the classical hypothalamic–pituitary–gonadal (HPG) axis. Overall, our findings provide new evidence supporting the role of the melatonin system in reproductive regulation in marine teleosts.

Keywords: melatonin receptors; reproduction; *Larimichthys crocea*; GnRH; HPG axis

Key Contribution: This study investigated the spatiotemporal expression of melatonin and its receptors in *Larimichthys crocea*. The results indicated that melatonin was primarily synthesized in the pineal gland, while the five melatonin receptors were predominantly expressed in the brain and testes. Especially, the expression of melatonin receptor genes in the testes was consistently higher than in the ovary throughout reproductive development, with a significant reduction in receptor expression in the testes during the later stages of reproductive development. In addition to modulating GnRH secretion in the brain, melatonin was also found to directly affect testosterone secretion in in vitro-incubated testes, with these effects being concentration-dependent.

Academic Editor: Juan F. Asturiano

Received: 4 December 2024
Revised: 5 January 2025
Accepted: 9 January 2025
Published: 10 January 2025

Citation: Liang, X.; Wang, J.; Huang, B.; Yuan, H.; Ren, Y.; Wu, C.; Wang, T.; Yang, J. Functional Involvement of Melatonin and Its Receptors in Reproductive Regulation of the Marine Teleost, Large Yellow Croaker (*Larimichthys crocea*). *Fishes* **2025**, *10*, 28. https://doi.org/10.3390/fishes10010028

1. Introduction

Melatonin, or N-acetyl-5-methoxytryptamine, is primarily synthesized in the pineal gland and released mainly during the dark phase of the circadian cycle [1,2]. Melatonin, first identified in 1958, is structurally similar to serotonin [3,4]. While the pineal gland is its

main source, melatonin is also secreted by other tissues such as the liver and enterochromaffin cells in rodents [5,6] and the retina in some fish species [7,8]. Key rate-limiting enzymes, Arylalkylamine N-acetyltransferase (AANAT) and N-acetylserotonin O-methyltransferase (ASMT), mediate melatonin synthesis by responding to photic and non-photic signals [9,10]. Current research indicates that melatonin regulates diverse physiological processes, including circadian rhythm, growth, reproduction, and antioxidation in fish [11–13].

Melatonin's effects are mainly mediated through receptors with a characteristic 7-transmembrane structure belonging to the rhodopsin-like (Class A) family of G protein-coupled receptors (GPCRs) [14]. In fish, these receptors are classified into four subtypes: *MTNR1A* (*MT1*, *Mel1a*), *MTNR1B* (*MT2*, *Mel1b*), *MTNR1C* (*Mel1c*, *GPR50* in mammals), and the recently reported *MTNR1D* (*Mel1d*) [15,16]. These receptor subtypes vary in tissue distribution, ligand affinity, and signaling properties [17]. While *MTNR1A* and *MTNR1B* are widely expressed across tissues, *MTNR1C* is generally less prevalent, primarily found in the skin and retina in certain fish species [18,19]. *MTNR1D*, yet to be fully understood, is largely expressed in the brain, diencephalon, eyes, and skin in species like puffer fish and medaka [17,20]. MTNR1C shows the highest melatonin affinity (MTNR1C > MTNR1A > MTNR1B > MTNR1D) [21,22]. Activation of these receptors involves different G proteins, with MTNR1A often coupling with $G_{\alpha i}/G_{\alpha s}$ to regulate cyclic adenosine monophosphate (cAMP) levels [23–25] and MTNR1B coupling with $G_{\alpha q}$ to increase intracellular Ca^{2+} or with $G_{\alpha t}$ to decrease cyclic guanosine monophosphate (cGMP) [26,27]. MTNR1C and MTNR1D predominantly engage with $G_{\alpha i}$, reducing cAMP [22,28].

Reproductive development, a fundamental biological process, is intricately regulated by the neuroendocrine system [29]. Melatonin and its receptors play an essential role in synchronizing physiological and behavioral processes, including reproduction, with responses to daily or seasonal photoperiod changes [30,31]. Its reproductive regulation is mainly achieved via the hypothalamic–pituitary–gonadal (HPG) axis, modulating steroid hormone levels. For example, in zebrafish (*Danio rerio*), melatonin stimulates the gene transcription of gonadotropin-releasing hormones (GnRH), luteinizing hormones (LH), and follicle-stimulating hormones (FSH) [32,33], while in Nile tilapia (*Oreochromis niloticus*) and European sea bass (*Dicentrarchus labrax*), it suppresses hormone secretion [34,35]. Additionally, melatonin interacts with other key reproductive regulators, such as gonadotropin inhibiting hormones (GnIH) and Kisspeptin, mediating reproduction in goldfish (*Carassius auratus*) and zebrafish [33,36,37]. In addition, in addition to the HPG axis pathway, melatonin could also directly act on the gonads to regulate physiological functions, for instance, it influences on ovarian development in sheep and bovine [38,39] and testicular development in mice [40,41]. Testosterone, a key hormone primarily produced by the testes in males, plays a vital role in reproductive function. Melatonin has been shown to enhance testosterone synthesis in mammalian Leydig cells. [42,43]. In fish, while the relationship between the melatonin system and reproduction is well established [12], the underlying regulatory models and networks can be further elucidated.

The large yellow croaker (*Larimichthys crocea*) is a commercially vital marine species in China, yet its wild populations have drastically declined [44]. Understanding its reproductive regulation mechanisms is essential for effective conservation and breeding efforts. However, current research on its reproduction is limited, focusing primarily on gender differentiation and control [45]. This study highlights the role of melatonin in regulating fish reproduction, providing a theoretical basis for advancing aquaculture practices.

2. Materials and Methods

2.1. Animal Collection

Various developmental stages of the same batch of healthy *L. crocea* were collected from the Administration of Fishery Research Institute of Zhoushan in Zhejiang Province. All fish were maintained in seawater with controlled temperature (22–23 °C), oxygen levels (8.0 ± 0.3 mg/L), and salinity (28–30‰). The fish of different magnitudes, of which the number was more than 120, were cultured in seven breeding tanks (104 cm in diameter, 113 cm in height, 500 L water) for at least 2 weeks before the experiment. Based on previous studies, the sexual maturation process in fish is classified into five stages (I to V, also designated as M1 to M5 for males and F1 to F5 for females) [46]. In this study, samples of *L. crocea* were categorized into three stages: stage II (M2, F2), stage III (M3, F3), and stage IV (M4, F4).

2.2. Samples Preparation

Before all fish were dissected, 0.01% Tricaine methanesulfonate (MS-222, E10505, Sigma Aldrich. St. Louis, MO, USA) was used for anesthesia. *L. crocea* (length, stage II: 12 ± 1.5 cm, September 2023, stage III: 17 ± 2 cm, May 2024, stage IV: 33 ± 2.5 cm, February–November 2024) were dissected for genetic analysis, histological analysis, and hormone-related experiments. The whole brain (including hypothalamus (hyp) and pituitary (pit)), heart (hea), liver (liv), spleen (spl), testes (tes), ovary (ova), and intestine (int) were obtained and stored in liquid nitrogen for subsequent gene expression analysis. Respective tissues were fixed in 4% paraformaldehyde for histological analysis. The brain and testes were cultured in Leibovitz L-15 (PH: 7.2–7.4, Procell, Wuhan, China) for hormone level measurement. For the suppression experiment, tissues from fish (5.2 ± 0.3 cm) in the early developmental stage were pretreated with a melatonin inhibitor (Luzindole, a broad-spectrum melatonin receptor antagonist, 2×10^{-7} mol/L) for 2 h.

2.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted from small tissues using the RNAfast200 Total RNA Rapid Extraction Kit (Fei jie biological company, Shanghai, China). RNA concentration and quality were measured and evaluated using a Nanodrop 2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) by UV absorbance. Samples with an OD 260 nm/OD 280 nm ratio between 1.8 and 2.1 were selected. The PrimeScript RT reagent kit (TaKaRa, Kusatsu, Japan) was used to synthesize cDNA and the RNase inhibitor (Promega Inc., Shanghai, China) was added during the cDNA synthesis process. The cDNA samples were stored at −80 °C for subsequent experiments.

2.4. Analysis of qRT-PCR

The primers employed for PCR amplification were detailed in Table 1, and their specificity was first tested to ensure that a single discrete band was amplified without primer dimers. The qRT-PCR was conducted using the CFX Connect Real-Time PCR System (BIO-RAD, Singapore) with TB Green Premix Ex Taq™ (TaKaRa, Shiga, Japan), and *β-Tubulin* served as the internal reference gene. The expression variations in *ANNAT2* and melatonin receptors were subjected to normalization, and estimation was performed utilizing the $2^{-\Delta\Delta Ct}$ equation.

Table 1. List of primers used for qRT-PCR analysis.

Gene	Sequence (5′→3′)	Size (bp)
Lc MTNR1A1	F: TACAGGTGAGGAGACGAGTGAAG R: GAAGTAGGCCATGAAGTAGCTGG	215
Lc MTNR1A2	F: GTGAGCTCGCTGTACACTATCAC R: CGACGAACATGGTGAGGAAGTTG	181
Lc MTNR1B1	F: ACACAGTGGCAGTAGTAGTGGT R: CAGCACAAAGACCACGAACATG	179
Lc MTNR1B2	F: TCAACCGCTACTGTTACATCTGC R: GTATGAGGTGCTGACTGTCTGTG	194
Lc MTNR1C	F: ACATCTTCGTGGTGAGTTTGTCC R: GTGATGTTGAAGATGGAGCCGAT	169
Lc AANAT2	F: CACTTACTCACGTCTAACATGGA R: TTGTCCTTACACAGTCCTTCTTC	187
Lc GnRH3	F: AGCGAACCTTTTCTTTCGGT R: TCCTATGGATGGCTACCAGG	186
Lc β-Tubulin	F: TGCCTTCATGGTAGATAACGAGG R: TCAGATCAACATTGAGAGCACCA	158

2.5. Hematoxylin–Eosin (HE) Staining and Immunohistochemistry

Tissues (brain, testes, and ovary) were fixed overnight in 4% paraformaldehyde. Subsequently, they were dehydrated in an ethanol gradient at room temperature and resized for optimal preparation. Following impregnation with wax, tissues were embedded in paraffin (Solarbio, Beijing, China) and left overnight in a 4 °C refrigerator. Sectioning of the paraffin-embedded tissues was conducted using a pathological slicer (Leica Biosystems, Wetzler, Germany). Tissue sections were deparaffinized with xylene for 10 min twice, then sequentially rehydrated in ethanol at concentrations of 100%, 95%, 90%, and 75% for 2 min each. The sections were then stained with hematoxylin (Aangon Biotech, Shanghai, China) for 5 min, rinsed in distilled water for 1 min, treated with 1% hydrochloric acid ethanol for 30 s, gently washed under tap water for 15 min, stained with eosin (Aangon Biotech, Shanghai, China) for 2 min, and rinsed again in distilled water for 1 min. Finally, the sections were mounted with neutral resin (Solarbio, Beijing, China, 96949-21-2).

The organ sections of the brain, testes, and ovary (5 μm thick) were dewaxed and rehydrated using xylene and ethanol gradients. Antigen retrieval was performed in a microwave (95–100 °C) using antigen retrieval solution. Subsequently, blocking was conducted for 1 h using a blocking solution, followed by overnight incubation at 4 °C with anti-melatonin receptor 1A (1:500, AB203038, Abcam, Cambridge, Britain) and anti-melatonin receptor 1B (1:250, AB203346, Abcam). This was followed by a 2 h incubation with Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (H+L) (1:500, A0423, Beyotime, Shanghai, China). DAPI (Beyotime, Shanghai, China) was then applied for a 30 min nuclear counterstain, and coverslips were sealed with glycerol. The control group was treated identically to the experimental group, except that primary antibodies were replaced with an antibody dilution buffer. Image acquisition was performed using an inverted fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). The binding efficiency of the antibodies mentioned above in *L. crocea* has been validated by previous experiments [28].

2.6. Measurement of Gonadotropin-Releasing Hormone and Testosterone

The hormone levels of GnRH released by the brain and testosterone released by the gonads were detected by competitive inhibition method. The enzyme-linked immunosor-

bent commercial assay kits (GnRH and testosterone) used in this experiment were from Nanjing Jiancheng Bioengineering Institute. The brain and testes obtained by dissection were placed in L-15 medium with different concentrations of melatonin (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} mol/L). The tissues were then cultured for 5 h (brain) and 4 h (testes) at 25 °C in an incubator. According to the kit instructions, the tissues were homogenized using the weight (g): volume (mL) ratio of 1:9 PBS buffer (PH 7.4), and the samples were homogenized fully. Centrifuged at 2000–3000 rpm/min for 20 min, the supernatant was collected. After packaging, some were used for detection, and the rest were frozen at −80 °C. Subsequent experimental procedures were conducted following the specifications of the assay kits. The absorbance of the sample was measured at a wavelength of 450 nm.

2.7. Statistical Analysis

Data analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Before the application of analysis of variance, all data were subjected to homogeneity of variance and homogeneity of distribution analysis. Differences between groups were examined through one-way analysis of variance (ANOVA) and Tukey's post hoc test. A significance threshold of $p < 0.05$ has been set. Significance levels are indicated by lowercase letters (a, b, c, etc.). Different letters indicate significant differences between groups, while the same letters indicate no significant differences between groups. The data were presented as mean ± standard deviation (SD).

3. Result

3.1. Expression of AANAT2 in Different Tissues of L. crocea

To identify key tissues involved in melatonin synthesis, the expression levels of *AANAT2*, a rate-limiting enzyme in melatonin synthesis, were analyzed across tissues.

The results revealed that *AANAT2* expression was significantly higher in the pineal gland than in other tissues ($p < 0.05$). Notably, higher expression levels were also observed in the heart, spleen, and intestine (Figure 1).

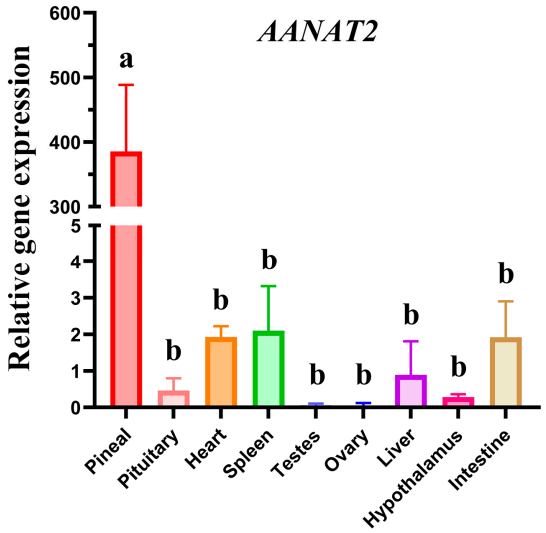

Figure 1. Expression of *AANAT2* in different tissues of *Larimichthys crocea* (stage II). The levels of the respective mRNAs were determined using qRT-PCR and normalized to the house keeping gene (*β-Tubulin*). Each vertical bar represents the mean ± S.D. (n = 3). Different letters on the column indicate statistically significant differences ($p < 0.05$).

3.2. Expression Characteristics of LcMTNRs Subtypes in L. crocea

The qRT-PCR analysis revealed high expression of five melatonin receptors in the pituitary gland (Figure 2A–E). *LcMTNR1B1* was also detected at moderate levels in the hypothalamus, while *LcMTNR1C* showed high expression in the testes, suggesting its potential role in regulating male reproduction. Notably, significant differences in the expression of all five receptors were observed between male and female gonads. Compared to the testes and brain, expression levels in other tissues, such as the liver and spleen, were significantly lower. These findings were consistent with those obtained through PCR analysis (Figure 2F).

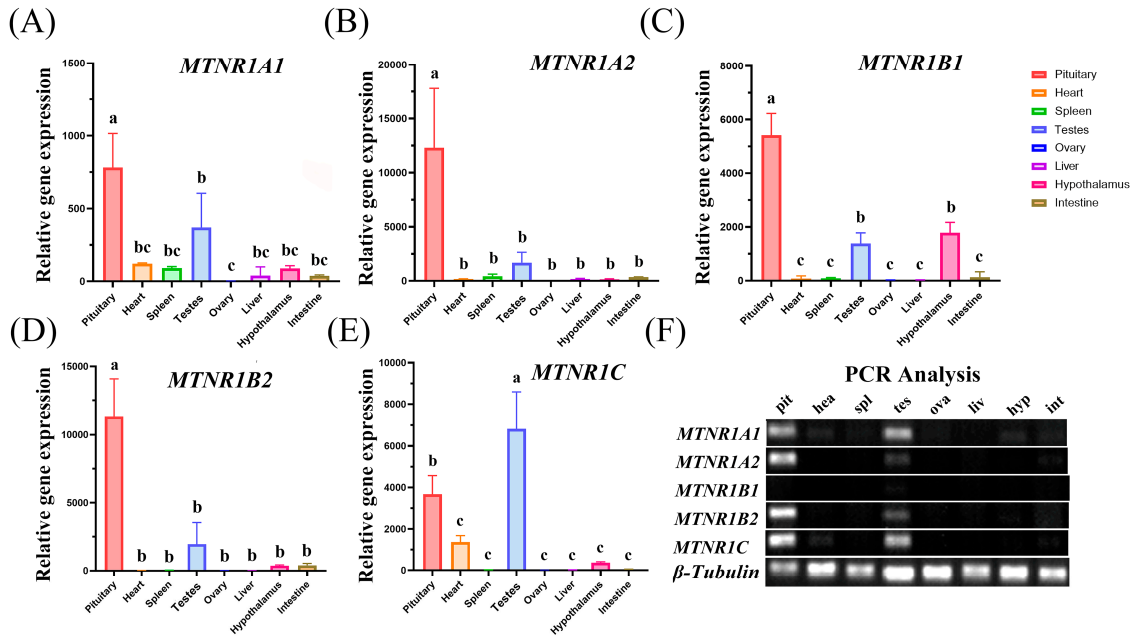

Figure 2. Distribution of five *LcMTNRs* mRNA in different tissues of *Larimichthys crocea* (stage II). (**A**–**E**) Relative expression of *LcMTNRs* in multiple tissues of *L. crocea* (hypothalamus, pituitary, heart, liver, spleen, testes, ovary, and intestine). The expression value was normalized against the expression of the internal control gene (*β-Tubulin*). Each vertical bar represents the mean ± S.D. (n = 3). Different lowercase letters above the bars indicate significant differences between different tissues ($p < 0.05$). (**F**) PCR analysis of *LcMTNRs* expression in multiple tissues (pit: pituitary, hea: heart, spl: spleen, tea: teatis, ova: ovary, liv: liver, hyp: hypothalamus, int: intestine).

3.3. Verification of Melatonin Receptor Expression in the Brain and Gonads

The immunohistochemistry results showed that *MTNR1A* and *MTNR1B* were primarily expressed in the brain and testes of *L. crocea*, with no expression detected in the ovary (Figure 3). The two melatonin receptors were indicated by green fluorescence from Alexa Fluor 488, and the nuclei were labeled with blue fluorescence from DAPI. In negative controls, where no primary antibodies were applied, fluorescence was not detected, confirming the specificity and effectiveness of the antibodies. These results align with the quantitative results.

3.4. Determination of Different Reproductive Periods in L. crocea

The *L. crocea* at various reproductive stages were collected and classified using hematoxylin and eosin (HE) staining. Reproductive stages were determined according to criteria from previous studies [46,47], based on the morphology, number, and size of gametocytes. The developmental process is generally categorized into five stages (I to V), with M1 to M5 and F1 to F5 denoting the respective stages for males and females. In this study, the fish

were grouped into three reproductive phases: stage II (M2 and F2, developing), stage III (M3 and F3, maturing), stage IV (M4 and F4, ripe) (Figure 4).

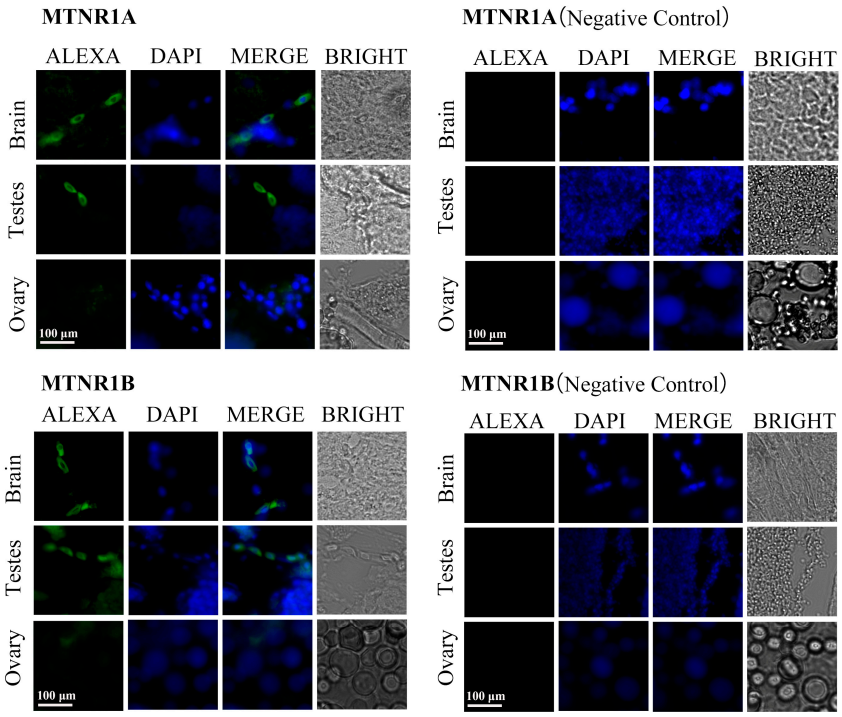

Figure 3. Distribution of MTNR1A and MTNR1B analyzed by immunohistochemistry in brain, testes, and ovary of *Larimichthys crocea* (stage II). Immuno-labeled MTNRs (green) in relative tissue is shown by the representative photomicrographs. "ALEXA" indicates that tissues were incubated with anti-MTNRs antibody and bonded with the corresponding secondary antibody. "DAPI" indicates nuclear counterstain. "Negative Control" indicates sections were incubated with antibody dilution buffer instead of primary antibody (scale = 100 μm).

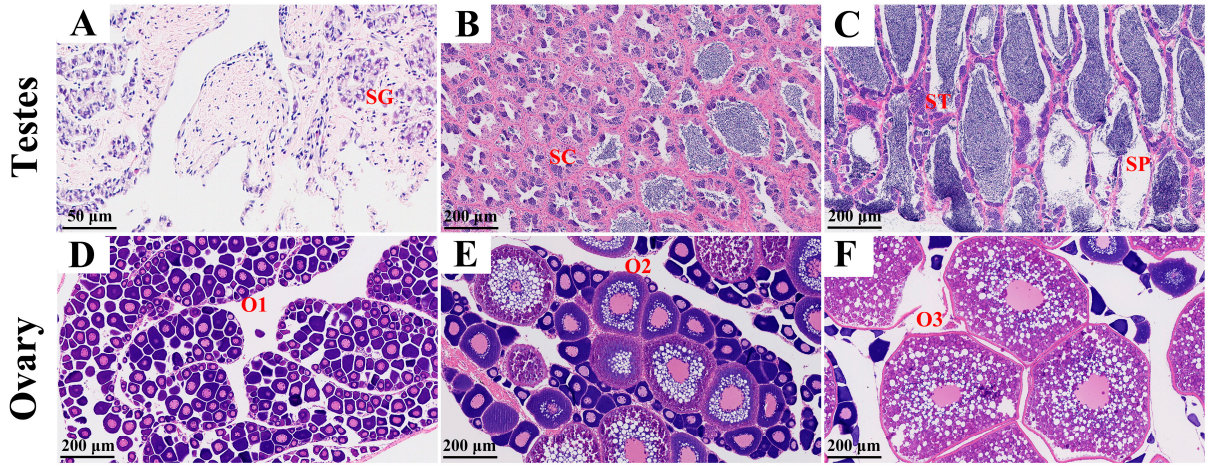

Figure 4. Histological sections of male and female gonads from different reproductive stages of *Larimichthys crocea*. The testes of *L. crocea*, respectively, in M2 (**A**), M3 (**B**), and M4 (**C**), the ovaries of *L. crocea*, respectively, F2 (**D**), F3 (**E**), and F4 (**F**). Typically categorized into five phases (I to V, M1 to M5 represent the phases in male and F1 to F5 represent the female). SG: Spermatogonia, SC: Spermatocytes, ST: spermatids, SP: Sperm, O1: Primary growth stage oocyte, O2: Cortical alveolar stage oocytes, O3: Vitellogenic stage oocytes.

3.5. Expression of LcMTNRs Across Different Reproductive Phases

The expression patterns of five melatonin receptors were analyzed across various tissues, including the brain, testes, ovary, and heart, during different reproductive phases. During stage II (Figure 5A), all five receptor pairs showed elevated expression in brain, with *MTNR1A2*, *MTNR1B1*, and *MTNR1B2* being the most prominent. Notably, *MTNR1C* showed high expression in testes, even surpassing its levels in the brain. In contrast, receptor expression in heart tissue was minimal and almost absent in the ovary. During stage III (Figure 5B), receptor expression remained stable in the brain but increased significantly in the testes, with *MTNR1A2*, *MTNR1B2*, and *MTNR1C* surpassing brain levels. A notable upregulation of receptor expression was also observed in the heart, while expression in the ovary remained minimal. During stage IV (Figure 5C), melatonin receptor expression remained elevated in the brain, with a reduced disparity between the gonads. However, the gene expression in testes still exceeded that in ovary. Additionally, heart tissue showed further increases, with *MTNR1A2* and *MTNR1C* levels surpassing those in brain.

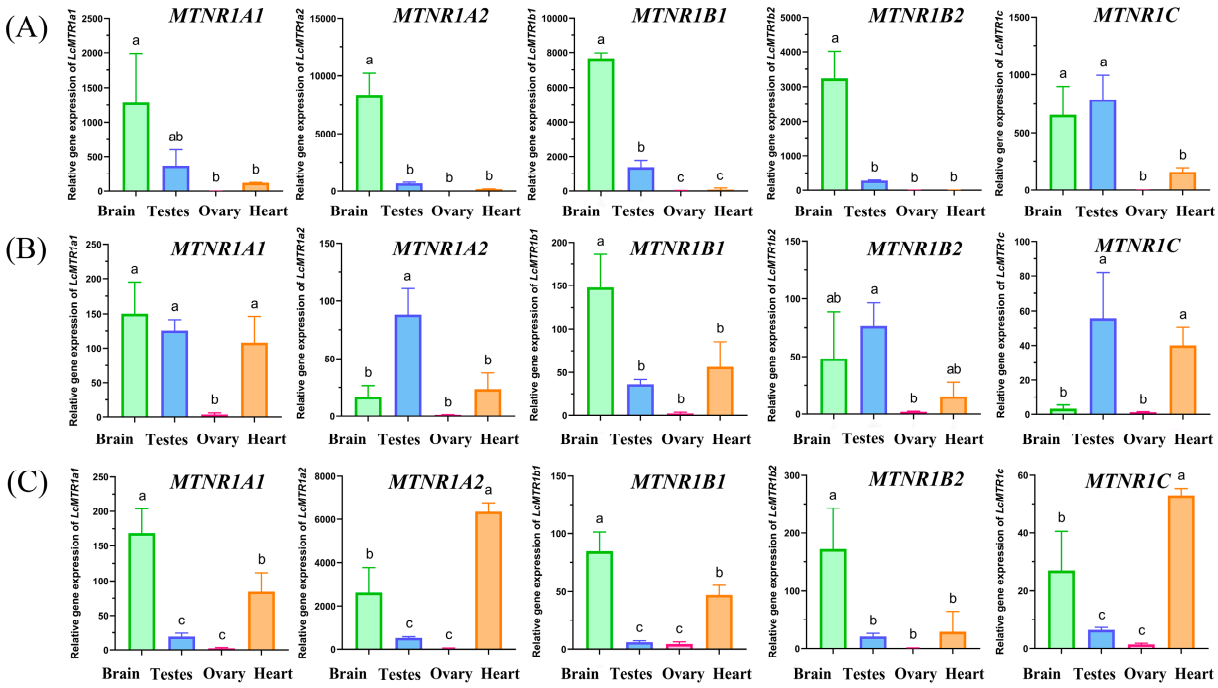

Figure 5. Characteristics of *LcMTNRs* expression in different reproductive stages. The expression of melatonin receptors in the brain, testes, ovary, and heart of *Larimichthys crocea* in stage II (**A**), stage III (**B**) and stage IV (**C**). The different letters of all on the column indicate statistically significant differences ($p < 0.05$, n = 3).

3.6. Impact of Melatonin on Gene Expression and Secretion of GnRH in the Brain

We conducted in vitro incubation experiments on fish (5.2 ± 0.3 cm) in the early developmental stage to assess *GnRH3* gene expression in the brain via qRT-PCR. Melatonin at a concentration of 10^{-10} mol/L significantly inhibited *GnRH3* expression. Blocking experiments with 2×10^{-7} mol/L Luzindole reversed melatonin-induced inhibition (Figure 6A,B). Additionally, in vitro incubation was performed on two groups of *L. crocea* in stage II and stages IV. In developing individuals (stage II, 12 ± 1.5 cm), melatonin at low concentrations stimulated GnRH secretion in the brain, peaking at 10^{-10} mol/L, with higher concentrations (notably 10^{-6} mol/L) showing inhibitory effects ($p < 0.05$) (Figure 6C). In contrast, melatonin did not elicit significant concentration-dependent effects on individuals (stage IV, 33 ± 2.5 cm) (Figure 6D).

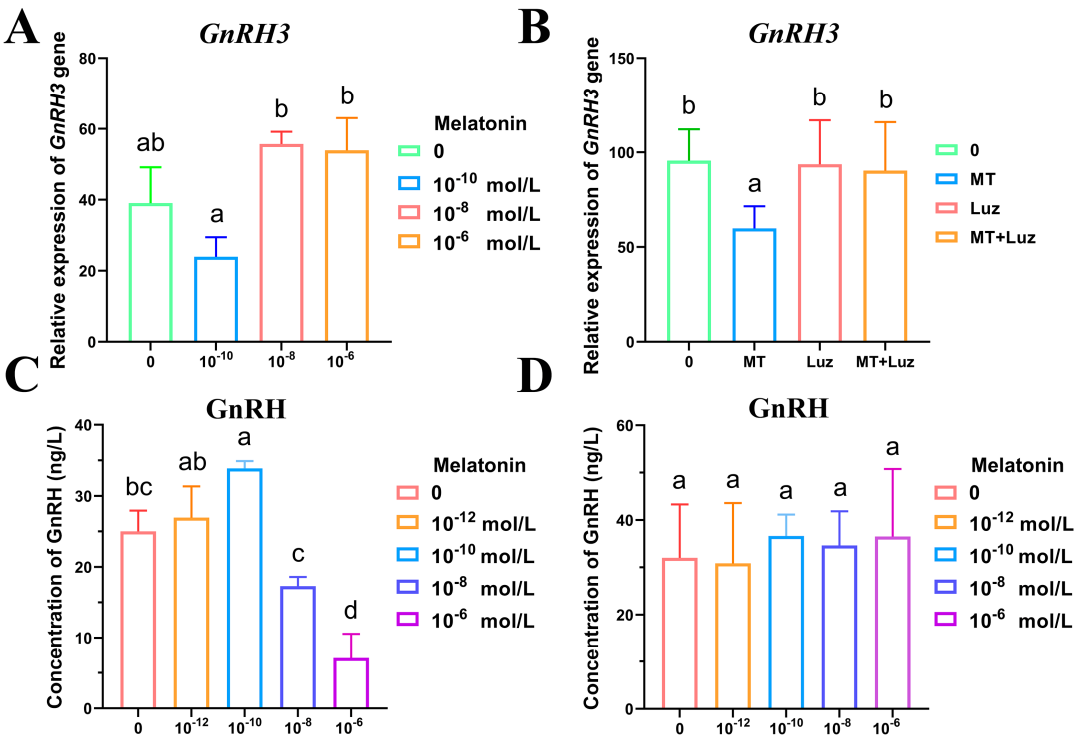

Figure 6. The effect of melatonin treatment on *GnRH* expression and secretion in the brain. (**A**) Expression of *GnRH3* gene of *Larimichthys crocea* (5.2 ± 0.3 cm) after treatment with different concentrations of melatonin for 2 h, (**B**) expression of *GnRH3* gene of *L. crocea* (5.2 ± 0.3 cm) after treatment with melatonin (MT) and luzindole (Luz, a broad-spectrum melatonin receptor antagonist) for 2 h. The secretion of GnRH in brain of *L. crocea* in stage II (**C**) and stage IV (**D**) after treatment with different concentrations of melatonin for 5 h. The different letters on the column indicate statistically significant differences ($p < 0.05$, n =3).

3.7. Impact of Melatonin on Testosterone Secretion in the Testes

In vitro testes incubation showed that melatonin significantly enhanced testosterone secretion at a concentration of 10^{-10} mol/L in *L. crocea* (stage II). However, higher concentrations (10^{-8} to 10^{-6} mol/L) inhibited testosterone secretion (Figure 7A). In contrast, melatonin inhibited testosterone secretion at nearly all concentrations in *L. crocea* (stage IV), with the inhibition intensifying as the concentration increased (Figure 7B).

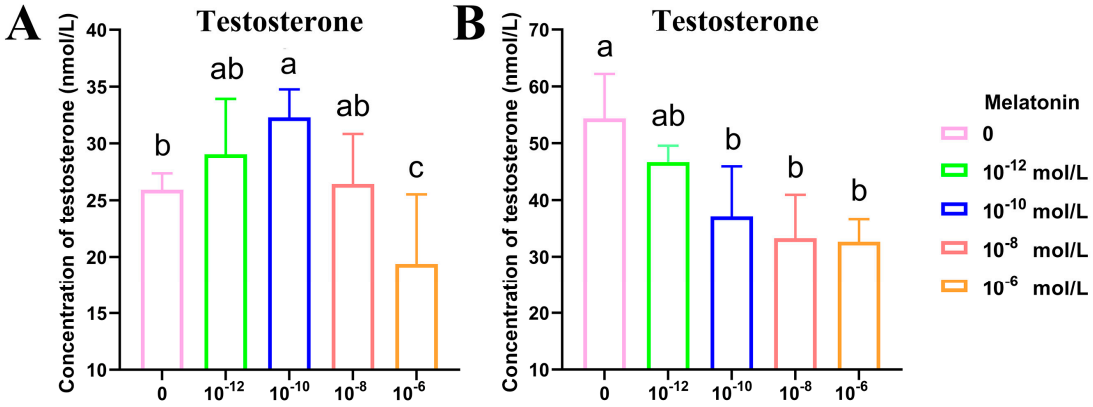

Figure 7. The effect of melatonin treatment on testosterone secretion in the testes. Testosterone secretion in the testes after treatment with different concentrations of melatonin for 4 h to the fish in stage II (**A**) and stage IV (**B**). ($p < 0.05$, n = 4).

4. Discussion

GnRH is a key hormone regulating reproduction, released by the hypothalamus in a pulsatile manner. Variations in GnRH pulse frequency and amplitude modulate the synthesis and release of FSH and LH [48]. Our findings demonstrated that in *L. crocea*, melatonin influenced GnRH secretion, an effect inhibited by melatonin receptor antagonists. This conclusion was consistent with findings from other studies [49,50]. In particular, GnRH secretion in the brain was significantly affected during stage II: as melatonin levels increased, GnRH secretion initially rose and then declined. However, this concentration-dependent inhibitory effect of melatonin was not observed in ripe fish (stage IV). These results indicated that melatonin played a critical role in reproductive function and gonadal development, with effects mediated by its concentration. Our study provides novel insights into this relationship, though further investigations are required to elucidate the underlying mechanisms.

The expression of *AANAT2*, a key gene in melatonin synthesis, was determined with significantly higher expression in the pineal gland compared to other tissues [51], consistent with findings in zebrafish [52]. Higher expression levels in the heart, spleen, and intestine suggest potential extra-pineal sites for melatonin synthesis. The high levels of melatonin receptors expression in the brain across different stages were observed, indicating that melatonin primarily exerted its effects in the central nervous system. These results align with other studies [53,54].

The high expression of *MTNRs* genes in the testes, as confirmed by immunohistochemistry, is similar to the study in the wild roe and the ram [55,56]. It was noteworthy that the expression of *MTNRs* in the testes showed variation during reproductive development, with a marked increase during the stage III, followed by a decrease in stage IV. This suggests that melatonin may be involved in sexual maturity. Melatonin receptors have been identified in various reproductive organs, including the ovary of goldfish [57], and the testes of Atlantic salmon (*Salmo salar*) [18], supporting the hypothesis that melatonin influences gonadal function via a pathway distinct from the conventional HPG axis, warranting further exploration. Furthermore, we found significant differences in melatonin receptor expression between the testes and ovary of *L. crocea*, a trend that persisted throughout the examined reproductive cycle. In previous studies, gender differences in melatonin binding sites were discovered [58]. *MTNRs* expression in testes was significantly higher than in the ovary, a pattern similar to that observed in synchronizing turbot (*Scophthalmus Maximus*) [59]. As development progressed, *MTNRs* expression in the testes increased initially then decreased, while expression in the ovary remained consistently low. This contrasts with findings in turbot, where testicular *MTNRs* expression decreased before increasing, and also differs from studies on Senegalese sole (*Solea senegalensis*) [60], European sea bass [18], and mudskippers [54], which reported a gradual decline in *MTNRs* expression during reproductive development. These interspecies variations may arise from differences in reproductive stage definitions and inherent species differences. Additionally, the disparities of melatonin receptor expression between male and female gonads may relate to gender developmental differences.

Our results also showed that *MTNR1C* was the key receptor with the highest relative expression in the testes among the five melatonin receptors in *L. crocea*, and its expression changed significantly across reproductive stages. Although, the melatonin binding ability and pharmacological properties of *MTNR1C* have been studied [61–63], research on *MTNR1C* in fish is still relatively limited and its potential relationship with reproduction was being reported for the first time. This finding provided clues and a reference for future studies to further validate and explore the important role of this receptor in fish reproductive regulation. Unexpectedly, the heart, initially selected as a control tissue

due to its relatively low melatonin receptor expression, exhibited a gradual increase in receptor expression during stages III and stage IV. In contrast, expression levels in the testes progressively declined, with the heart eventually surpassing the testes by stage IV. Although further research is needed, this suggests that melatonin's role may shift from regulating reproduction to metabolism as reproductive maturation advances.

Moreover, the results of this study demonstrated that melatonin treatment significantly influenced testosterone secretion during both stages II and IV. Previous studies have reported that melatonin regulates the synthesis of testosterone; however, its effects vary among species [64,65]. In our results, the altered testosterone induces by melatonin, combined with the high expression of melatonin receptors in the testes of *L. crocea*, suggested that melatonin signals were transduced via an endocrine pathway, implicating its role in reproductive regulation. Previous studies have shown that melatonin directly affects ovarian function in fish. For instance, short-term melatonin exposure through injection or immersion decreases the gonadosomatic index in *Channa punctatus* [66], while removal of melatonin-secreting organs, such as the pineal gland or eyes, disrupts ovarian development [67]. However, direct interactions between melatonin and the testes in teleost require further validation.

5. Conclusions

In conclusion, this study confirmed the high expression of *AANAT2* in *L. crocea* pineal gland, with five melatonin receptors primarily located in the pituitary and gonads, showing sexual gender differences: *MTNRs* were expressed in the testes but nearly absent in the ovary. This suggested that, beyond the classical HPG axis pathway, melatonin might directly activate gonadal receptors to regulate reproduction. Receptor expression peaked in early stages to stage III then declining in stage IV, indicating that melatonin's regulatory role is concentrated before stage IV. Additionally, melatonin incubation showed a concentration-dependent inhibition of GnRH secretion in the brain at 10^{-10}–10^{-12} mol/L, with no significant effect at higher concentrations. The above preliminary results suggested melatonin's regulatory effect on fish reproduction was condition-specific, varying by sex, timing, and concentration. These findings provide a foundation for investigating the connection between rhythmic patterns and reproductive processes in teleost fish, offering meaningful insights into melatonin's diverse roles in fish reproduction for future research.

Author Contributions: Conceptualization, J.Y. and T.W.; investigation, X.L. and J.W.; data curation, X.L., B.H. and H.Y.; validation, J.Y.; formal analysis, Y.R. and C.W.; resources, X.L. and J.W.; writing—original draft preparation, X.L.; writing—review and editing, X.L., J.W. and J.Y.; project administration, J.Y; funding acquisition, T.W. and J.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Natural Science Foundation of China (Nos. 42076130), National Key Research and Development Program of China (2023YFD2401905), and the Fundamental Research Funds for Zhejiang Provencial Universities and Research Institutes (2024J002).

Institutional Review Board Statement: The procedures for care and use of animals were approved by the Ethics Committee of Zhejiang Ocean University and all applicable institutional and governmental regulations concerning the ethical use of animals were followed (ethic code: 2024111, approval date: 20 August 2024).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: The authors of this paper would like to thank Weiye Li and Xiaolong Yin from Zhoushan Fishery Research Institute for fish sampling.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Claustrat, B.; Brun, J.; Chazot, G. The Basic Physiology and Pathophysiology of Melatonin. *Sleep. Med. Rev.* **2005**, *9*, 11–24. [CrossRef] [PubMed]
2. REITER, R.J. Pineal Melatonin: Cell Biology of Its Synthesis and of Its Physiological Interactions. *Endocr. Rev.* **1991**, *12*, 151–180. [CrossRef]
3. McCord, C.P.; Allen, F.P. Evidences Associating Pineal Gland Function with Alterations in Pigmentation. *J. Exp. Zool.* **1917**, *23*, 207–224. [CrossRef]
4. Lerner, A.B.; Case, J.D.; Takahashi, Y.; Lee, T.H.; Mori, W. Isolation of Melatonin, The Pineal Gland Factor that Lightens Melanocytes [1]. *J. Am. Chem. Soc.* **1958**, *80*, 2587. [CrossRef]
5. Sato, K.; Meng, F.; Francis, H.; Wu, N.; Chen, L.; Kennedy, L.; Zhou, T.; Franchitto, A.; Onori, P.; Gaudio, E.; et al. Melatonin and Circadian Rhythms in Liver Diseases: Functional Roles and Potential Therapies. *J. Pineal Res.* **2020**, *68*, e12639. [CrossRef]
6. Yang, C.; Lu, Z.; Xia, Y.; Zhang, J.; Zou, Z.; Chen, C.; Wang, X.; Tian, X.; Cheng, S.; Jiang, X. Alterations of Gut-Derived Melatonin in Neurobehavioral Impairments Caused by Zinc Oxide Nanoparticles. *Int. J. Nanomed.* **2023**, *18*, 1899–1914. [CrossRef]
7. Zawilska, J.B.; Lorenc, A.; Berezińska, M.; Vivien-Roels, B.; Pévet, P.; Skene, D.J. Diurnal and Circadian Rhythms in Melatonin Synthesis in the Turkey Pineal Gland and Retina. *Gen. Comp. Endocrinol.* **2006**, *145*, 162–168. [CrossRef] [PubMed]
8. Besseau, L.; Vuilleumier, R.; Sauzet, S.; Boeuf, G.; Falcón, J. Contrôle Photopériodique de La Synthèse de Mélatonine Par La Rétine et l'épiphyse de Poisson [Photoperiodic control of melatonin synthesis in fish pineal and retina]. *J. Soc. Biol.* **2007**, *201*, 13–20. [CrossRef] [PubMed]
9. Falcón, J.; Galarneau, K.M.; Weller, J.L.; Ron, B.; Chen, G.; Coon, S.L.; Klein, D.C. Regulation of Arylalkylamine *N*-Acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the Fish Pineal Organ: Evidence for a Role of Proteasomal Proteolysis. *Endocrinology* **2001**, *142*, 1804–1813. [CrossRef] [PubMed]
10. Tan, D.-X.; Manchester, L.; Esteban-Zubero, E.; Zhou, Z.; Reiter, R. Melatonin as a Potent and Inducible Endogenous Antioxidant: Synthesis and Metabolism. *Molecules* **2015**, *20*, 18886–18906. [CrossRef]
11. Prendergast, B.J. MT1 Melatonin Receptors Mediate Somatic, Behavioral, and Reproductive Neuroendocrine Responses to Photoperiod and Melatonin in Siberian Hamsters (*Phodopus sungorus*). *Endocrinology* **2010**, *151*, 714–721. [CrossRef] [PubMed]
12. Maitra, S.K.; Hasan, K.N. The Role of Melatonin as a Hormone and an Antioxidant in the Control of Fish Reproduction. *Front. Endocrinol.* **2016**, *7*, 38. [CrossRef] [PubMed]
13. Bano-Otalora, B.; Madrid, J.A.; Rol, M.A. Melatonin Alleviates Circadian System Disruption Induced by Chronic Shifts of the Light-dark Cycle in *Octodon degus*. *J. Pineal Res.* **2020**, *68*, e12619. [CrossRef] [PubMed]
14. Jockers, R.; Delagrange, P.; Dubocovich, M.L.; Markus, R.P.; Renault, N.; Tosini, G.; Cecon, E.; Zlotos, D.P. Update on Melatonin Receptors: IUPHAR Review 20. *Br. J. Pharmacol.* **2016**, *173*, 2702–2725. [CrossRef]
15. Dufourny, L.; Levasseur, A.; Migaud, M.; Callebaut, I.; Pontarotti, P.; Malpaux, B.; Monget, P. GPR50 Is the Mammalian Ortholog of Mel1c: Evidence of Rapid Evolution in Mammals. *BMC Evol. Biol.* **2008**, *8*, 105. [CrossRef] [PubMed]
16. Denker, E.; Ebbesson, L.O.E.; Hazlerigg, D.G.; Macqueen, D.J. Phylogenetic Reclassification of Vertebrate Melatonin Receptors to Include Mel1d. *G3 Genes Genomes Genet.* **2019**, *9*, 3225–3238. [CrossRef]
17. Maugars, G.; Nourizadeh-Lillabadi, R.; Weltzien, F.-A. New Insights into the Evolutionary History of Melatonin Receptors in Vertebrates, With Particular Focus on Teleosts. *Front. Endocrinol.* **2020**, *11*, 538196. [CrossRef]
18. Sauzet, S.; Besseau, L.; Herrera Perez, P.; Covès, D.; Chatain, B.; Peyric, E.; Boeuf, G.; Muñoz-Cueto, J.A.; Falcón, J. Cloning and Retinal Expression of Melatonin Receptors in the European Sea Bass, *Dicentrarchus labrax*. *Gen. Comp. Endocrinol.* **2008**, *157*, 186–195. [CrossRef] [PubMed]
19. Falcón, J.; Migaud, H.; Muñoz-Cueto, J.A.; Carrillo, M. Current Knowledge on the Melatonin System in Teleost Fish. *Gen. Comp. Endocrinol.* **2010**, *165*, 469–482. [CrossRef] [PubMed]
20. Ikegami, T.; Motohashi, E.; Doi, H.; Hattori, A.; Ando, H. Synchronized Diurnal and Circadian Expressions of Four Subtypes of Melatonin Receptor Genes in the Diencephalon of a Puffer Fish with Lunar-Related Spawning Cycles. *Neurosci. Lett.* **2009**, *462*, 58–63. [CrossRef]
21. Dubocovich, M.L.; Markowska, M. Functional MT_1 and MT_2 melatonin receptors in mammals. *Endocrine* **2005**, *27*, 101–110. [CrossRef]
22. Sakai, K.; Yamamoto, Y.; Ikeuchi, T. Vertebrates Originally Possess Four Functional Subtypes of G Protein-Coupled Melatonin Receptor. *Sci. Rep.* **2019**, *9*, 9465. [CrossRef]

23. Chen, L.; He, X.; Zhang, Y.; Chen, X.; Lai, X.; Shao, J.; Shi, Y.; Zhou, N. Melatonin Receptor Type 1 Signals to Extracellular Signal-Regulated Kinase 1 and 2 via G_i and G_s Dually Coupled Pathways in HEK-293 Cells. *Biochemistry* **2014**, *53*, 2827–2839. [CrossRef]
24. Cecon, E.; Oishi, A.; Jockers, R. Melatonin Receptors: Molecular Pharmacology and Signalling in the Context of System Bias. *Br. J. Pharmacol.* **2018**, *175*, 3263–3280. [CrossRef] [PubMed]
25. Ciani, E.; Fontaine, R.; Maugars, G.; Mizrahi, N.; Mayer, I.; Levavi-Sivan, B.; Weltzien, F. Melatonin Receptors in Atlantic Salmon Stimulate CAMP Levels in Heterologous Cell Lines and Show Season-dependent Daily Variations in Pituitary Expression Levels. *J. Pineal Res.* **2019**, *67*, e12590. [CrossRef] [PubMed]
26. Petit, L.; Lacroix, I.; de Coppet, P.; Strosberg, A.D.; Jockers, R. Differential Signaling of Human Mel1a and Mel1b Melatonin Receptors through the Cyclic Guanosine 3′-5′-Monophosphate Pathway. *Biochem. Pharmacol.* **1999**, *58*, 633–639. [CrossRef] [PubMed]
27. Okamoto, H.H.; Cecon, E.; Nureki, O.; Rivara, S.; Jockers, R. Melatonin Receptor Structure and Signaling. *J. Pineal Res.* **2024**, *76*, e12952. [CrossRef] [PubMed]
28. Feng, J.; Yang, J.; Jiang, Z.; Zhou, N.; Liu, X.; Zhang, G.; Yan, X.; Wang, J.; Xu, X.; Guo, S.; et al. Melatonin Modulates the Hypothalamic-Pituitary Neuroendocrine Axis to Regulate Physiological Color Change in Teleost Fish. *Int. J. Biol. Sci.* **2023**, *19*, 2914–2933. [CrossRef] [PubMed]
29. Rocha, R.M.P.; Lima, L.F.; Alves, A.M.C.V.; Celestino, J.J.H.; Matos, M.H.T.; Lima-Verde, I.B.; Bernuci, M.P.; Lopes, C.A.P.; Báo, S.N.; Campello, C.C.; et al. Interaction between Melatonin and Follicle-Stimulating Hormone Promotes in Vitro Development of Caprine Preantral Follicles. *Domest. Anim. Endocrinol.* **2013**, *44*, 1–9. [CrossRef]
30. Falcón, J.; Besseau, L.; Fuentès, M.; Sauzet, S.; Magnanou, E.; Boeuf, G. Structural and Functional Evolution of the Pineal Melatonin System in Vertebrates. *Ann. N. Y. Acad. Sci.* **2009**, *1163*, 101–111. [CrossRef]
31. Borjigin, J.; Samantha Zhang, L.; Calinescu, A.-A. Circadian Regulation of Pineal Gland Rhythmicity. *Mol. Cell. Endocrinol.* **2012**, *349*, 13–19. [CrossRef] [PubMed]
32. Carnevali, O.; Gioacchini, G.; Maradonna, F.; Olivotto, I.; Migliarini, B. Melatonin Induces Follicle Maturation in *Danio rerio*. *PLoS ONE* **2011**, *6*, e19978. [CrossRef] [PubMed]
33. Yumnamcha, T.; Khan, Z.A.; Rajiv, C.; Devi, S.D.; Mondal, G.; Sanjita Devi, H.; Bharali, R.; Chattoraj, A. Interaction of Melatonin and Gonadotropin-Inhibitory Hormone on the Zebrafish Brain-Pituitary-Reproductive Axis. *Mol. Reprod. Dev.* **2017**, *84*, 389–400. [CrossRef] [PubMed]
34. Servili, A.; Herrera-Pérez, P.; Del Carmen Rendón, M.; Muñoz-Cueto, J. Melatonin Inhibits GnRH-1, GnRH-3 and GnRH Receptor Expression in the Brain of the European Sea Bass, *Dicentrarchus labrax*. *Int. J. Mol. Sci.* **2013**, *14*, 7603–7616. [CrossRef] [PubMed]
35. Kim, J.-H.; Park, J.W.; Jin, Y.H.; Kim, D.-J.; Kwon, J.Y. Effect of Melatonin on GnIH Precursor Gene Expression in Nile Tilapia, *Oreochromis niloticus*. *Biol. Rhythm. Res.* **2018**, *49*, 303–313. [CrossRef]
36. Choi, C.Y.; Shin, H.S.; Kim, N.N.; Yang, S.-G.; Kim, B.-S.; Yu, Y.M. Time-Related Effects of Various LED Light Spectra on Reproductive Hormones in the Brain of the Goldfish *Carassius auratus*. *Biol. Rhythm. Res.* **2015**, *46*, 671–682. [CrossRef]
37. Tsutsui, K.; Ubuka, T. Discovery of Gonadotropin-Inhibitory Hormone (GnIH), Progress in GnIH Research on Reproductive Physiology and Behavior and Perspective of GnIH Research on Neuroendocrine Regulation of Reproduction. *Mol. Cell. Endocrinol.* **2020**, *514*, 110914. [CrossRef] [PubMed]
38. Cavalcante, B.N.; Matos-Brito, B.G.; Paulino, L.R.F.M.; Silva, B.R.; Aguiar, A.W.M.; de Almeida, E.F.M.; Souza, A.L.P.; Vasconcelos, G.L.; De Assis, E.I.T.; Silva, A.W.B.; et al. Effects of Melatonin on Morphology and Development of Primordial Follicles during in Vitro Culture of Bovine Ovarian Tissue. *Reprod. Domest. Anim.* **2019**, *54*, 1567–1573. [CrossRef] [PubMed]
39. Ma, J.; Wang, J.; Hu, S.; Li, Y.; Zhang, Y.; Yang, Y.; Yang, C.; Huo, S.; Yang, Y.; Zhaxi, Y.; et al. Effects of Melatonin on Development and Hormone Secretion of Sheep Theca Cells in Vitro. *Theriogenology* **2023**, *198*, 172–182. [CrossRef]
40. Yu, K.; Deng, S.-L.; Sun, T.-C.; Li, Y.-Y.; Liu, Y.-X. Melatonin Regulates the Synthesis of Steroid Hormones on Male Reproduction: A Review. *Molecules* **2018**, *23*, 447. [CrossRef]
41. Kasahara, T.; Abe, K.; Mekada, K.; Yoshiki, A.; Kato, T. Genetic Variation of Melatonin Productivity in Laboratory Mice under Domestication. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 6412–6417. [CrossRef] [PubMed]
42. Yang, M.; Guan, S.; Tao, J.; Zhu, K.; Lv, D.; Wang, J.; Li, G.; Gao, Y.; Wu, H.; Liu, J.; et al. Melatonin Promotes Male Reproductive Performance and Increases Testosterone Synthesis in Mammalian Leydig Cells. *Biol. Reprod.* **2021**, *104*, 1322–1336. [CrossRef] [PubMed]
43. Liu, X.; Guo, X.; Zhang, T.; Duan, J.; Zhang, L.; Wang, M.; Li, Y.; Shen, Z.; Mao, J. Testosterone Maintains Male Longevity and Female Reproduction in *Chrysopa pallens*. *Heliyon* **2024**, *10*, e32478. [CrossRef] [PubMed]
44. Zhang, G.; Ye, Z.; Jiang, Z.; Wu, C.; Ge, L.; Wang, J.; Xu, X.; Wang, T.; Yang, J. Circadian Patterns and Photoperiodic Modulation of Clock Gene Expression and Neuroendocrine Hormone Secretion in the Marine Teleost *Larimichthys crocea*. *Chronobiol. Int.* **2024**, *41*, 329–346. [CrossRef] [PubMed]

45. Xiao, J.; Cao, K.; Zou, Y.; Xiao, S.; Wang, Z.; Cai, M. Sex-Biased Gene Discovery from the Gonadal Transcriptomes of the Large Yellow Croaker (*Larimichthys crocea*). *Aquac. Fish.* **2019**, *4*, 9–16. [CrossRef]
46. Yan, L.; Jiang, Y.; Xu, Q.; Ding, G.; Chen, X.; Liu, M. Reproductive Dynamics of the Large Yellow Croaker *Larimichthys crocea* (Sciaenidae), A Commercially Important Fishery Species in China. *Front. Mar. Sci.* **2022**, *9*, 8580. [CrossRef]
47. Zhang, L.L.; Liu, M.; Fang, L.P.; Xu, Q.; Lin, J.J. Reproductive Biology of *Johnius taiwanensis* (Perciformes: Sciaenidae) in Fujian Waters, Southern China. *Zool. Stud.* **2019**, *58*, e38. [CrossRef]
48. Stamatiades, G.A.; Kaiser, U.B. Gonadotropin Regulation by Pulsatile GnRH: Signaling and Gene Expression. *Mol. Cell. Endocrinol.* **2018**, *463*, 131–141. [CrossRef]
49. Zhang, W.; Zhang, Z.; Peng, J.; Yang, S.; Tong, D. Effects of Melatonin on the Production of GnRH and LH in Luteal Cells of Pregnant Sows. *J. Mol. Endocrinol.* **2022**, *68*, 111–123. [CrossRef] [PubMed]
50. Ding, M.; Jiang, S.; Miao, J.; Pan, L. Possible Roles of Gonadotropin-Releasing Hormone (GnRH) and Melatonin in the Control of Gonadal Development of Clam *Ruditapes philippinarum*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2021**, *262*, 111059. [CrossRef] [PubMed]
51. Wang, Y.; Zhang, Z.; Guo, H.; Li, P.; Li, J.; Xi, L.; Liu, Q. Stimulation Role of Epinephrine in Transcription of the Melatonin Synthesis Key Enzyme AANAT in the Pineal Gland of Broilers. *Mol. Cell. Biochem.* **2019**, *453*, 111–119. [CrossRef]
52. Gothilf, Y.; Coon, S.L.; Toyama, R.; Chitnis, A.; Namboodiri, M.A.A.; Klein, D.C. Zebrafish Serotonin N-Acetyltransferase-2: Marker for Development of Pineal Photoreceptors and Circadian Clock Function1. *Endocrinology* **1999**, *140*, 4895–4903. [CrossRef] [PubMed]
53. Park, Y.-J.; Park, J.-G.; Hiyakawa, N.; Lee, Y.-D.; Kim, S.-J.; Takemura, A. Diurnal and Circadian Regulation of a Melatonin Receptor, MT1, in the Golden Rabbitfish, *Siganus guttatus*. *Gen. Comp. Endocrinol.* **2007**, *150*, 253–262. [CrossRef] [PubMed]
54. Hong, L.Y.; Hong, W.S.; Zhu, W.B.; Shi, Q.; You, X.X.; Chen, S.X. Cloning and Expression of Melatonin Receptors in the Mudskipper *Boleophthalmus pectinirostris*: Their Role in Synchronizing Its Semilunar Spawning Rhythm. *Gen. Comp. Endocrinol.* **2014**, *195*, 138–150. [CrossRef] [PubMed]
55. Kozioł, K.; Broda, D.; Romerowicz-Misielak, M.; Nowak, S.; Koziorowski, M. Melatonin Concentration in Peripheral Blood and Melatonin Receptors (MT1 and MT2) in the Testes and Epididymis of Male Roe Deer during Active Spermatogenesis. *Theriogenology* **2020**, *149*, 25–37. [CrossRef] [PubMed]
56. González-Arto, M.; Aguilar, D.; Gaspar-Torrubia, E.; Gallego, M.; Carvajal-Serna, M.; Herrera-Marcos, L.; Serrano-Blesa, E.; Hamilton, T.; Pérez-Pé, R.; Muiño-Blanco, T.; et al. Melatonin MT1 and MT2 Receptors in the Ram Reproductive Tract. *Int. J. Mol. Sci.* **2017**, *18*, 662. [CrossRef]
57. Ikegami, T.; Azuma, K.; Nakamura, M.; Suzuki, N.; Hattori, A.; Ando, H. Diurnal Expressions of Four Subtypes of Melatonin Receptor Genes in the Optic Tectum and Retina of Goldfish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2009**, *152*, 219–224. [CrossRef] [PubMed]
58. Menendez-Pelaez, A.; Lopez-Gonzalez, M.A.; Guerrero, J.M. Melatonin Binding Sites in the Harderian Gland of Syrian Hamsters: Sexual Differences and Effect of Castration. *J. Pineal Res.* **1993**, *14*, 34–38. [CrossRef] [PubMed]
59. Zhao, C.; Xu, S.; Liu, Y.; Feng, C.; Xiao, Y.; Wang, Y.; Liu, Q.; Li, J. Changes of Melatonin and Its Receptors in Synchronizing Turbot (*Scophthalmus maximus*) Seasonal Reproduction and Maturation Rhythm. *Acta Oceanol. Sin.* **2022**, *41*, 84–98. [CrossRef]
60. Confente, F.; Rendón, M.C.; Besseau, L.; Falcón, J.; Muñoz-Cueto, J.A. Melatonin Receptors in a Pleuronectiform Species, Solea Senegalensis: Cloning, Tissue Expression, Day–Night and Seasonal Variations. *Gen. Comp. Endocrinol.* **2010**, *167*, 202–214. [CrossRef]
61. Reppert, S.M.; Weaver, D.R.; Cassone, V.M.; Godson, C.; Kolakowski, L.F. Melatonin Receptors Are for the Birds: Molecular Analysis of Two Receptor Subtypes Differentially Expressed in Chick Brain. *Neuron* **1995**, *15*, 1003–1015. [CrossRef]
62. Pickering, H.; Sword, S.; Vonhoff, S.; Jones, R.; Sugden, D. Analogues of Diverse Structure Are Unable to Differentiate Native Melatonin Receptors in the Chicken Retina, Sheep Pars Tuberalis and *Xenopus* Melanophores. *Br. J. Pharmacol.* **1996**, *119*, 379–387. [CrossRef]
63. Jockers, R.; Petit, L.; Lacroix, I.; Coppet, P.d.; Barrett, P.; Morgan, P.J.; Guardiola, B.; Delagrange, P.; Marullo, S.; Strosberg, A.D. Novel Isoforms of Mel1c Melatonin Receptors Modulating Intracellular Cyclic Guanosine 3′,5′-Monophosphate Levels. *Mol. Endocrinol.* **1997**, *11*, 1070–1081. [CrossRef] [PubMed]
64. Carvajal-Serna, M.; Neira-Rivera, E.; Cardozo, J.A.; Grajales-Lombana, H.; Cebrián-Pérez, J.Á.; Muiño-Blanco, T.; Pérez-Pé, R.; Casao, A. Melatonin Membrane Receptors MT1 and MT2 Are Expressed in Ram Spermatozoa from Non-Seasonal Breeds. *Trop. Anim. Health Prod.* **2020**, *52*, 2549–2557. [CrossRef] [PubMed]
65. Xu, H.; Pu, J.; Teng, Y.; Zhu, Q.; Guo, L.; Zhao, J.; Ding, H.; Fang, Y.; Ma, X.; Liu, H.; et al. Melatonin Inhibits Testosterone Synthesis in Rooster Leydig Cells by Targeting CXCL14 through MiR-7481-3p. *Int. J. Mol. Sci.* **2023**, *24*, 16552. [CrossRef] [PubMed]

66. Renuka, K.; Joshi, B.N. Melatonin-Induced Changes in Ovarian Function in the Freshwater Fish Channa Punctatus (Bloch) Held in Long Days and Continuous Light. *Gen. Comp. Endocrinol.* **2010**, *165*, 42–46. [CrossRef]
67. Garg, S.K. Effect of Pinealectomy, Eye Enucleation, and Melatonin Treatment on Ovarian Activity and Vitellogenin Levels in the Catfish Exposed to Short Photoperiod or Long Photoperiod. *J. Pineal Res.* **1989**, *7*, 91–104. [CrossRef] [PubMed]