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Expression and Characterization of the *Spats1* Gene and Its Response to E2/MT Treatment in the Chinese Soft-Shelled Turtle (*Pelodiscus sinensis*)

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Simple Summary: Spermatogenesis in *Pelodiscus sinensis* has distinct seasonal characteristics. *Spats1* acts a key function in germ cell differentiation in mammals, yet its study in *P. sinensis* has not been reported. Here, we cloned the coding sequences (CDS) of *Spats1* for the first time and characterized its expression in different tissues. In addition, the expression characteristics of *Spats1* in the testis during different seasons and the expression pattern in embryos and adults after steroid hormone treatment were also investigated. The present research provides a preliminary study on the physiological regulation of *Spats1* in *P. sinensis*, and lays the foundation for further systematic investigation of the molecular mechanisms of spermatogenesis and release in *P. sinensis*.

Abstract: *Spats1* (spermatogenesis-associated, serinerich 1) has been characterized as a male-biased gene which acts an important role in the germ cell differentiation of mammals. Nevertheless, the function of *Spats1* in the Chinese soft-shelled turtle (*P. sinensis*) has not yet been reported. To initially explore the expression of *Spats1* in *P. sinensis* and its response to sex steroid treatment, we cloned the CDS of *Spats1* for the first time and analyzed its expression profile in different tissues, including the testes in different seasons. The *Spats1* cDNA fragment is 1201 base pairs (bp) in length and contains an open reading frame (ORF) of 849 bp, which codes for 283 amino acids. *Spats1* mRNA was highly expressed in the testes ($p < 0.01$) and barely detectable in other tissues. In *P. sinensis*, the relative expression of *Spats1* also responsive to seasonal changes in testis development. In summer (July) and autumn (October), *Spats1* gene expression was significantly higher in the testes than in other seasons ($p < 0.05$). *Spats1* mRNA was found to be specifically expressed in germ cells by chemical in situ hybridization (CISH), and it was mainly located in primary spermatocytes (Sc1), secondary spermatocytes (Sc2) and spermatozoa (St). *Spats1* expression in embryos was not significantly changed after 17α-methyltestosterone (MT)and 17β-estradiol (E2) treatment. In adults, MT significantly induced *Spats1* expression in male *P. sinensis*. However, the expression of *Spats1* in testes was not responsive to E2 treatment. In addition, the expression of *Spats1* in females was not affected by either MT or E2 treatment. These results imply that *Spats1* is a male-specific expressed gene that is mainly regulated by MT and is closely linked to spermatogenesis and release in *P. sinensis*.

Keywords: cloning; expression; *Pelodiscus sinensis*; *Spats1*; steroid hormone

1. Introduction

Spermatogenesis is a complex physiological process that mainly includes spermatogonia cell differentiation and sperm formation [1]. Spermatogenesis occurs continuously in majority of mammals and birds without any relation to the season. However, spermatogenesis in reptiles is closely associated with the seasons [2], such as in wall lizard (*Podarcis muralis*) [3], Asian yellow pond turtles (*Mauremys mutica*) [4], and tropical rattlesnake (*Crotalus durissus*) [5]. *P. sinensis* is an important species of aquaculture which is broadly spread in Asia. Since the fast growth of turtle farming, the economic value of *P. sinensis* has also increased [6]. Phylogenetic analysis revealed that *P. sinensis* is a relatively ancient tetrapod, and the study of the morphological evolution and reproductive patterns of the species is of great scientific value [7,8]. In *P. sinensis*, spermatogenesis is a seasonally dependent physiological process which starts in the spring and proceeds through the summer and fall. Spermatogenesis occurs in May, and mature sperm is released at the end of October [9,10]. From December to the following March, *P. sinensis* enters hibernation, wherein sperm production is inactive [11]. Currently, studies on spermatogenesis of *P. sinensis* are mostly carried out by a histological examination [12], while less research has been conducted at the molecular level.

Sex hormones normally act as essential regulators in gonadal differentiation and sexual activity. In many species, the direction of sexual differentiation and the expression patterns of some genes related to sexual activity are modified by the treatment of sex steroid hormones [13,14]. In *P. sinensis*, sex steroid hormone treatment leads to sex reversal and affects the expression patterns of some genes that are closely related to sex differentiation. For example, significant masculinization of genetic female embryos and feminization of genetic male embryos were observed following aromatase inhibitor (AI) and E2 treatment. E2 could significantly downregulate the mRNA and protein expressions of doublesex-and-mab-3-related transcription factor 1 (*Dmrt1*) and SRY-box transcription factor 9 (*Sox9*), while AI exerted the opposite effect [15]. Although the trend of *R-spondin 1* (*Rspo1*) expression was inconsistent across different periods of embryonic development, it was up- and down-regulated after exogenous estradiol and letrozole treatment, respectively [16].

Spermatogenesis is a complex process that involves a multiplicity of growth factors and cytokines [17]. *Spats1* (spermatogenesis-associated serine-rich 1) is a gene that is tightly associated with spermatogenesis in mammals [18]. It was established that *Spats1* is an evolutionarily conserved, testis-specific protein which is differentially expressed during rat male meiotic prophase [19], and the highest level of *Spats1* expression was detected in primary spermatocytes among the different spermatogenic cell types [20]. Furthermore, *Spats1* has been shown to be a highly phosphorylatable protein [21]. In addition, recent studies have identified *Spats1* as a new molecular target in colorectal cancer [22]. However, no reports on the *Spats1* in *P. sinensis* have been found.

Of interest, according to transcriptome data (Accession: PRJNA838782) of ovary and testis in the early development of *P. sinensis* [23], *Spats1* was characterized as a male-specific expressed gene. In the present study, we obtained cDNA fragments of the *Spats1* in *P. sinensis*, and explored the tissue distribution of *Spats1* and seasonal patterns in testes for the first time. Significantly, this study also explored for the first time the response of *Spats1* to E2 and MT treatment in the embryonic and adult gonads of *P. sinensis*. These findings contribute to the clarification of the physiological functions of *Spats1* in *P. sinensis*, providing new viewpoints to investigate the regulatory mechanism of *Spats1* on spermatogenesis in *P. sinensis*, and enriching the basic data for *Spats1* in reptiles.

2. Materials and Methods

2.1. Animals and Ethics Statement

All experimental *P. sinensis* adults and embryos were obtained from Caixing Industrial Co. (Huizhou, China). All experimental procedures were performed in accordance with the regulations for animal care of the Pearl River Fisheries Research Institute (Guangzhou, China).

2.2. Collection of Samples

2.2.1. Cloning and Tissue Distribution Expression

A total of six *P. sinensis* were used for cloning and tissue distribution expression. Healthy three-year-old *P. sinensis* females and males (*n* = 3 each) were anesthetized with an intraperitoneal injection of 0.05% MS-222 (20 mg/kg, Sigma, St. Louis, MO, USA), and sacrificed. The heart, liver, spleen, kidney, brain, muscle, ovary and testis were sampled. Subsequently, these tissues were washed slightly with 1× PBS in diethylpyrocarbonate (DEPC) water, and stored in liquid nitrogen for total RNA extractions.

2.2.2. Seasonal Expression and In Situ Hybridization

A total of 36 male *P. sinensis* were used for exploring *Spats1* expression in different seasons and localization in cells. Three healthy *P. sinensis* males ranging from 1 to 2 and 3-years-old were acquired each time in January, April, July, and October. Animals were anesthetized using 0.05% MS-222 (Sigma, St. Louis, MO, USA), and testes were collected and stored in liquid nitrogen for total RNA extractions. Another portion of the testis tissue was placed in 4% paraformaldehyde (PFA) at 4 °C for overnight fixation. Subsequently, it was dehydrated using a methanol gradient and finally kept in 100% methanol at −20 °C until use.

2.2.3. Hormone Treatment in Embryonic Development

A total of 300 fertilized eggs were collected, identified, and put into the incubator in the laboratory of the Pearl River Fisheries Research Institute during 12 h after spawning. According to the reasonable temperature range of incubation, referring to the previous studies [24–27], the incubation temperature was kept at 31 °C, and the humidity was held at 75% in this study. These 300 fertilized eggs were divided into three groups: the MT-treated group, the E2-treated group, and the control group. Referring to the research of Cai et al. [28], embryos were treated with E2 and MT. E2 and MT were solubilized in 95% ethanol at a concentration of 20 μg/μL, and 5 μL of the medication was applied to the eggshell topically in the adjacent areas of embryonic development at stages 15 and 16 (gonadal differentiation usually starts at Stage 17) [29]. The control group was processed with 5 μL of 95% ethanol. After hormone treatment, incubation conditions in the treatment and control groups remained at a temperature of 31 °C and 75% humidity. At stage 25, the gonads were rapidly isolated from the embryo (both kidneys and gonads) and then frozen and stored in liquid nitrogen for RNA extraction. DNA from other tissues was extracted with a kit (Mabio, Xiamen, China) and the gender of the embryos was determined through PCR amplification (primer ZHB1) [30].

2.2.4. Hormone Treatment in Adults

A total of 106 adult *P. sinensis* were used for hormone treatment, of which 53 were females and 53 were males. The gonads of three males and three females were randomly collected as a blank control group before hormone treatment. The remaining 50 females *P. sinensis* were equally divided into 2 groups (E2 treatment group, MT treatment group), and the remaining 50 males were also equally divided into 2 groups (E2 treatment group, MT treatment group), and injected with E2 and MT from the leg muscles at a concentration of 50 μg/μL and a dose of 10 mg/kg. The gonads of three females and three males were randomly collected from each group at 0 h, 6 h, 12 h, 24 h, 48 h, and 7 days after hormone treatment and placed in liquid nitrogen for total RNA extractions.

2.3. RNA Extraction and cDNA Synthesis

Total RNA from different tissues was extracted by RNA iso Plus (Takara, Beijing, China) according to the instructions. The quality of RNA was detected according to RNA electrophoresis (Bio-Rad, PowerPacTM, CA, USA) and NanoDrop 2000 Spectrophotometer (Thermo Fisher, NanoDropOne, MA, USA). Total RNA from different tissues was used

as the template and the first-strand cDNA was synthesized following the protocol of the reverse transcription kit (Takara, Beijing, China).

2.4. Cloning and Sequencing

The primers (PsSpats1-1F/PsSpats1-1R) (Table 1) for *Spats1* fragment amplification were designed based on RNA-Seq. The *Spats1* was amplified with Taq Enzyme PCR (Accurate Biology, Changsha, China) according to the instructions. This step used approximately 2 µg of cDNA from the testes of *P. sinensis* with the primers PsSpats1-1F and PsSpats1-1R. The PCR conditions were 94 °C for 30 s, 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 72 °C for 60 s, and 72 °C for 2 min, followed by cooling to 4 °C. Products were electrophoresed in 1.5% agarose gels. The target fragments were purified using a Universal DNA Purification Kit (TIANGEN, Beijing, China), and then ligated with the pMD19-T vector (Takara, China). Afterwards, the products were transformed into *E. coli* DH5α competent cells following the manual, and the recombinants were screened by blue–white color on LB plates containing ampicillin. Positive clones were identified by PCR. The PCR conditions were 94 °C for 30 s, 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 72 °C for 60 s, and 72 °C for 2 min. The sequences of all product were verified at Tianyi Huiyuan (Guangzhou, China).

Table 1. Primers used in experiments.

Name	Application	Sequences (5′—3′)	Amplification Efficiency	Product Size (bp)
PsSpats1-1F	Clone	CCCTGAATCATGACCCCTCA		1201
PsSpats1-1R	Clone	TCCATGGAAACAACCACTGTGAA		
PsSpats1-2F	Semi-Quantitative/qRT-PCR	AGCGAGGGAAGTAGCAT	98.25%	251
Ps Spats1-2R	Semi-Quantitative/qRT-PCR	GGAAGGTGAAAGGGAAAA		
Ps Spats1-3F	CISH	TAATACGACTCACTATAG GGCGAGCCGACCTTCTAC-CCA		869
Ps Spats1-3R	CISH	ATTTAGGTGACACTATAGAA TACTCTCTGTCCTTTCG-CATTA		
Eef1a-F	Semi-Quantitative/qRT-PCR	ACTCGTCCAACTGACAAGCCTC	100.73%	337
Eef1a-R	Semi-Quantitative/qRT-PCR	CACGGCGAACATCTTTCACAG		
ZHB1-F	Sex identification PCR	CTGGAAACAATATCATCGCCGAG		590
ZHB1-R	Sex identification PCR	TGTGTGCCGTGCCTGCGA		

2.5. Structural Analysis

All amino acid sequences were obtained from NCBI GenBank and analyzed by DNA-MAN 6.0. The segmentation of signal peptides is predicted with the SignalP-5.0 server program (http://www.cbs.dtu.dk//services/SignalP/index.php accessed on 6 June 2022). ClustalX 1.83 was used to perform amino acid multiple sequence alignment and the protein 3D model was derived by SWISS-MODEL server. A phylogenetic tree was modeled by maximum likelihood (ML) method of MEGA 7 and ClustalW (https://myhits.sib.swiss/cgi-bin/clustalw accessed on 6 June 2022) through amino acid sequences.

2.6. Cryostat Sections of the Gonad

The testes were collected in July from the three ages (1, 2, and 3 years) and fixed overnight at 4 °C in 4% PFA. The testes were immersed in 30% sugar-PBS buffer at 4 °C for 5 h after PBS washing. Samples were wrapped with O.T.C. (Tissue-Tek. Sakura Finetek USA, Inc., Torrance, CA, USA) and cut into 4-µm sections using a cryostat microtome (Leica,

Wetzlar, Germany). The tissue sections were placed on slides and subsequently stored at −80 °C. Frozen tissue sections are mounted on frosted glass slides (4951 PLUS-001E, Thermo Fisher, MA, USA) and held at −80 °C till the next step of the experiment.

2.7. In Situ Hybridization

The amplification product gained from primers (PsISH Spats1-3F and PsISH Spats1-3R) (Table 1) designed based on the *P. sinensis Spats1* cDNA sequence were ligated to the plasmid pMD19-T (Takara, Beijing, China). Plasmid extraction was performed on positive clones. The vector plasmid was linearized by restriction enzymes Apa I and Sac II, and precipitated overnight by addition of ethanol and sodium acetate. Wash the precipitate with 75% ethanol and subsequently solubilize it using DEPC water. Antisense and sense probes were synthesized with the digoxigenin RNA Labeling Kit (Roche, Basle, Switzerland), which uses T7 and Sp6 promoters. In situ hybridization was carried out following the protocol of the Reagent Kits (G3024) (Servicebio, Wuhan, China). BCIP/NBT chromogenic solution was used in Chemical in situ hybridization (CISH). Propidium iodide (PI) was used to stain nuclei. The sections were mounted with an anti-quenching mounting agent (Gold Antifade reagent, Invitrogen, Shanghai, China). Observations and photographs were taken using an Axio Observer Z1 microscope and an Axiocam 506 color imaging system (ZEISS, Oberkochen, Germany).

2.8. Semi-Quantitative PCR and Real-Time Quantitative PCR

Based on the *Spats1* cDNA sequence of *P. sinensis*, a pair of specific primers PsSpats1-2F and PsSpats1-2R were designed for the semi-quantitative PCR performed (Table 1). The PCR condition was 35 cycles at 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 60 s. The primer (*Eef1a*-F/*Eef1a*-R) (Table 1) was used as a control with a PCR amplification fragment of 252 bp. Separate amplification products with 1.5% agarose gel, stained with ethidium bromide, and photographed with the Alpha Innotech bioimaging system.

The qRT-PCR was performed using the same primers (PsSpats1-2F and PsSpats1-2R) (Table 1), and *Eef1a* was used as the internal reference gene. Follow the instructions of iTaq Universal SYBR Supermix (BIO-RAD, CA, USA) for qRT-PCR. The PCR volume was 20 μL, containing SYBR Supermix (10 μL), cDNA (1 μL, 100 ng), each primer (1 μL, 2 μM), and nuclease-free water (7 μL). Following PCR conditions were applied for all primers: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 55–60 °C for 15 s, and 72 °C for 15 s; melting curve analysis occurred at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Three replicate experiments were analyzed for each sample. The expression level of *Spats1* was normalized with the expression of *Eef1a*, then analyzed by the $2^{-\Delta Ct}$ method for the expression level of *Spats1*.

2.9. Statistical Analysis

Normality tests were first performed to ensure that the *t*-test was applicable to the data analysis of this experiment, followed by Student's *t*-test or one-way ANOVA using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). All values are presented as a mean ± SEM. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning and Sequence Analyses

The *Spats1* cDNA fragment is 1201 base pairs (bp) in length (Figure 1) and contains an open reading frame (ORF) of 849 bp, which encodes for a protein with 283 amino acids.

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   1    TAAGCTGTTCCAGCTGGCATAATTATCCCTGAATCATGACCCCTCACTCCTCCTGCACAT
  61    TGGTCTCTGGAGAGCTGAAGACCCCAGAGCGGAGCTGGGTGTCACAGAGATGTGATGAAAC
 121    ACCATGCCCTCCAAGAATAAAGCAGAACTTATCGGCAAGCGAGGGAAGTAGCATGGAAAAA
   1          M  P  S  K  N  K  A  E  L  I  A  S  E  G  S  S  M  E  K
 181    CCAGAGGGCAATTCTGCATCACAAGGGTCCCAAGTCAGTGAGCCTGTCTACCTTCCTGAG
  20     P  E  G  N  S  A  S  Q  G  S  Q  V  S  E  P  V  Y  L  P  E
 241    GTCAGGAGCCATACAATGAAATGCAACAGACTTAATGAGCATCAGCACACATCCCTTGAC
  40     V  R  S  H  T  N  K  C  N  R  L  N  E  H  Q  H  T  S  L  D
 301    AGCACAAGTCAAGACCGTCTCAGCCGACCTTCTACCCATTCTACAGAGACTTCTTGTTCT
  60     S  T  S  Q  D  R  L  S  R  P  S  T  H  S  T  E  T  S  C  S
 361    AACCATGAGCATGAACTGAAGTCCAGGCTTTCCCTTTCAGCCTTCCTCTCAGCACACTGAT
  80     N  H  E  H  E  L  K  S  R  L  S  L  S  P  S  S  Q  H  T  D
 421    GGTCCACTACCTGCAATCAAACCATCACCTGAAACCCAACAATTTATTATGCCTCCTGAA
 100     G  P  L  P  A  I  K  P  S  P  E  T  Q  Q  F  I  M  P  P  E
 481    AAGAAGAGGGACCCAGACTCAGAATGGACTTTTTATCCACGGTTTGGTCTTCACACATAT
 120     K  K  R  D  P  D  S  E  W  T  F  Y  P  R  F  G  L  H  T  Y
 541    CATATTGGCAAACGATGCTTCTTTGATGGTATCTTTCTCCGGAAACAAAACCTGTGCATCA
 140     H  I  G  K  R  C  F  F  D  G  I  F  L  R  N  K  T  C  A  S
 601    GAAAGGATGTTAGAAATGTGTTTGGGAAGAAAAAGACATGAAATTGATCAGAGAAATGGA
 160     E  R  M  L  E  M  C  L  G  R  K  R  H  E  I  D  Q  R  N  G
 661    ATTGCTCTCGGTGACCCCCGGGTCACAACAGCTACAGCTGTCCAGAACAAAGCAGAAACTTC
 180     I  A  L  V  T  P  G  D  N  S  Y  S  C  P  E  Q  S  R  N  F
 721    CACAAATTTGGATCAACGAGGCCTGTGGTGAACTTTGGGTGTGCTACATACAAAAGGAAA
 200     H  K  F  G  S  T  R  P  V  V  N  F  G  C  A  T  Y  K  R  K
 781    GCAGATACATTTATTCCTCTCCGGCACTTGTCAGAGACTCCTTGCATACCTTTCAACATG
 220     A  D  T  F  I  P  L  R  H  L  S  E  T  P  C  I  P  F  N  M
 841    AAGGAGAAACAGGAAGAATTGGAGACAGAGATGAACAATGTGAAAAGTCTAGAAAGATGG
 240     K  E  K  Q  E  E  L  E  T  E  M  N  N  V  K  S  L  E  R  W
 901    ACACCTGCTCCAACATTGATTCAGGCCTTTGGTGGCCACTGGCCTGGCACACAGAATCAAA
 260     T  P  A  P  T  L  I  Q  A  F  G  A  T  G  L  A  H  R  I  K
 961    CAGCCGTGAACCATAATGGAAAGTTCTTCCATTTTGTAAGCCACTCATATCTCTTGACGAC
 280     Q  R  E  P  *
1021    ATAACATACATATGCAAATACTTCTGGATGTGATTTCTTACTTGGACGGTCCTTGTAATG
1081    TGTTGTAGCAACCACTGTAGATAGTCTGTTTTCAGACTTTGTATTGACTTTAGTTCATGA
1141    TCCCACCTGAACAGAACAAAAATGTGTAGTTAATGCGAAAGGACAGAGTAGCTTTTTGCC
1201    CCACGTCATGTTACTTTCTTCACAGTGGTTGTTTCCATGGAATAAAGTTCTTGTG
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Figure 1. Nucleotide and predicted amino acid sequences of *P. sinensis Spats1.* The start codon (ATG) is underlined by a single line, and the stop codon (TAA) is represented by the asterisks.

The inferred molecular weight of this protein is 32.15 kDa, and shares a 73.78% identity with *Chelonoidis abingdonii* Spats1 (XP_032655998.1), 73.33% with *Terrapene carolina triunguis* Spats1 (XP_029767743.1), and 73.33% with *Mauremys reevesii* Spats1 (XP_039386593.1). Yet, the sequence had low homology with those of mammals, fish, and amphibians, such as *Homo sapiens* SPATS1 (NP_001359010.1) (45.08%), *Bufo bufo* Spats1 (XP_040286913.1) (26.32%), and *Danio rerio* Spats1 (XP_017208226.1) (19.67%) (Figure 2).

The protein 3D structures of human (SPATS1) and zebrafish (Spats1) are similar in that they both begin with two α-helices at the C-terminus and are followed by a certain number of shorter α-helices interconnected by disordered loops. Although the N-terminal part of Spats1 in *P. sinensis* also starts with two α-helices, the number of subsequent short α-helices is smaller than that in both human and zebrafish (Figure 3). To better illustrate the evolutionary relationships of the *Spats1* gene between different species, a phylogenetic tree was structured by maximum likelihood (ML) from the putative amino acid sequences of 11 species, inclusive of *P. sinensis*. The Spats1 proteins were classified into four clusters: mammalia, reptilia, amphibia, and teleosts; these clades were strongly supported by the bootstrap values (Figure 4). *P. sinensis* Spats1 was classified in the reptile cluster and was associated more strongly with Spats1 from turtles than from other species.

3.2. Tissue Distribution

Tissue distributions of *Spats1* in three-year-old *P. sinensis* specimens were analyzed by Semi-Quantitative PCR and qRT-PCR, using *Eef1a* as a reference gene. Gel electrophoresis maps showed bright amplified band of *Spats1* only in testis (Figure 5A). *Spats1* was detected to highly expressed in testes via qRT-PCR (DF = 13, F = 682.449, $p < 0.001$), but rarely found in other tissues (Figure 5B). Hence, it was demonstrated that *Spats1* mRNA was sex-specific expressed in the gonads and was only in the adult testes of *P. sinensis*.
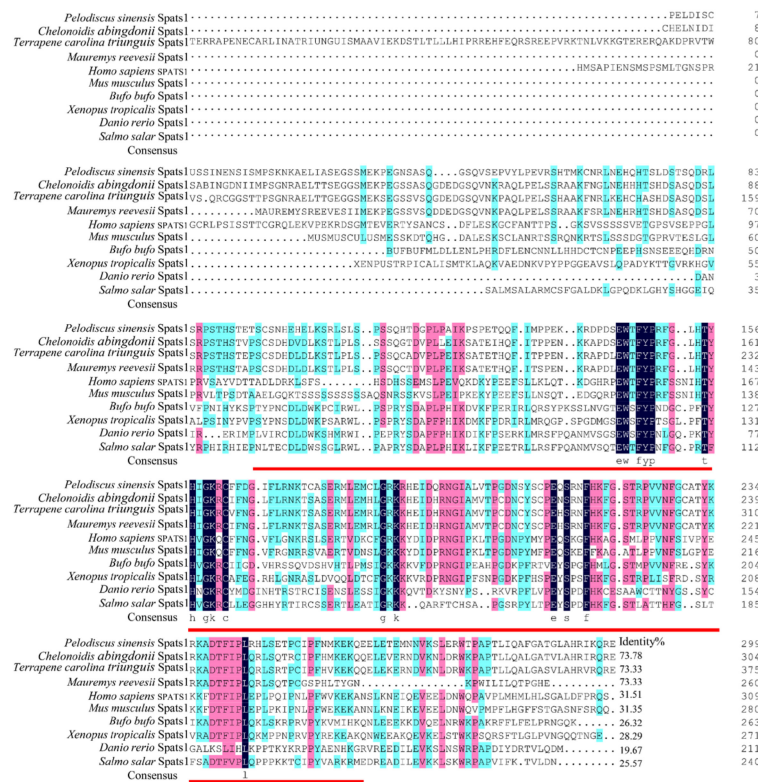
Figure 2. Alignment of the amino acid sequences. The structural domain is underlined in red. GenBank accession numbers: *Chelonoidis abingdonii* Spats1 (XP_032655998.1), *Terrapene carolina triunguis* Spats1 (XP_029767743.1), *Mauremys reevesii* Spats1 (XP_039386593.1), *Homo sapiens* SPATS1 (NP_001359010.1), *Bufo bufo* Spats1 (XP_040286913.1), *Danio rerio* Spats1 (XP_017208226.1), *Mus musculus* Spats1 (NP_081925.2), *Xenopus tropicalis* Spats1 (XP_002936034.2), and *Salmo salar* Spats1 (XP_014000180.1). Black shading indicates the same amino acid sequence. Pink shading indicates conserved sequences with more than 75% of the listed polypeptides. The blue shading indicates the conserved sequence with more than half of the listed peptides.
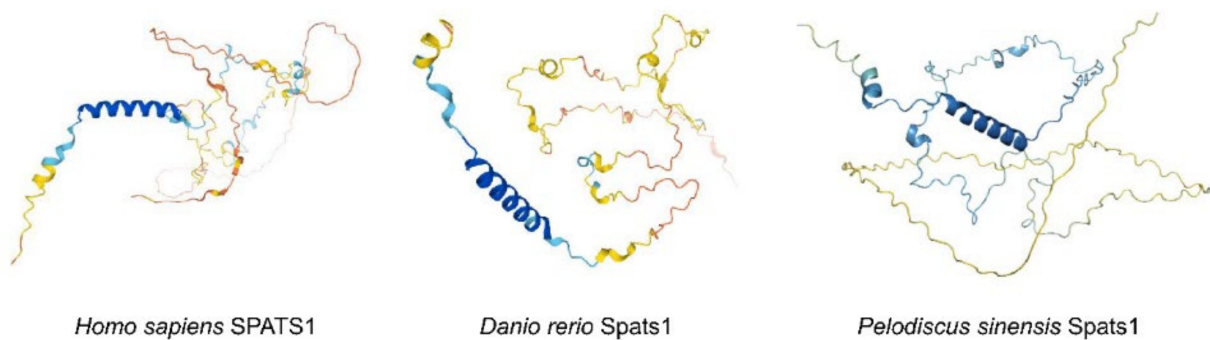

Figure 3. The protein 3D structure models. Different colors represent different model confdence. ■: Very high (Plddt > 90); ■: Confident (90 > Plddt > 70); ■: Low (70 > Plddt > 50); ■: Very low (Plddt < 50).

3.3. The Cellular Distribution of Spats1 mRNA in Testes

This is the first time that *Spats1* mRNA was localized in the male germ cells of 1-, 2-, and three-year-old *P. sinensis* specimens. The CISH of the testis sections revealed that chemical signals of *Spats1* transcripts were detected with the antisense probes (Figure 6A,C,E,G), while no signal was observed with sense probes (Figure 6B). The nucleus was stained with PI (Figure 6D,F,H). *Spats1* mRNA was mainly distributed in primary spermatocytes (Sc1),

secondary spermatocytes (Sc2), and spermatozoa (St). However, in spermatogonia (Sg) and somatic cells (*), *Spats1* expression was weakly (Figure 6A,C,E,G).
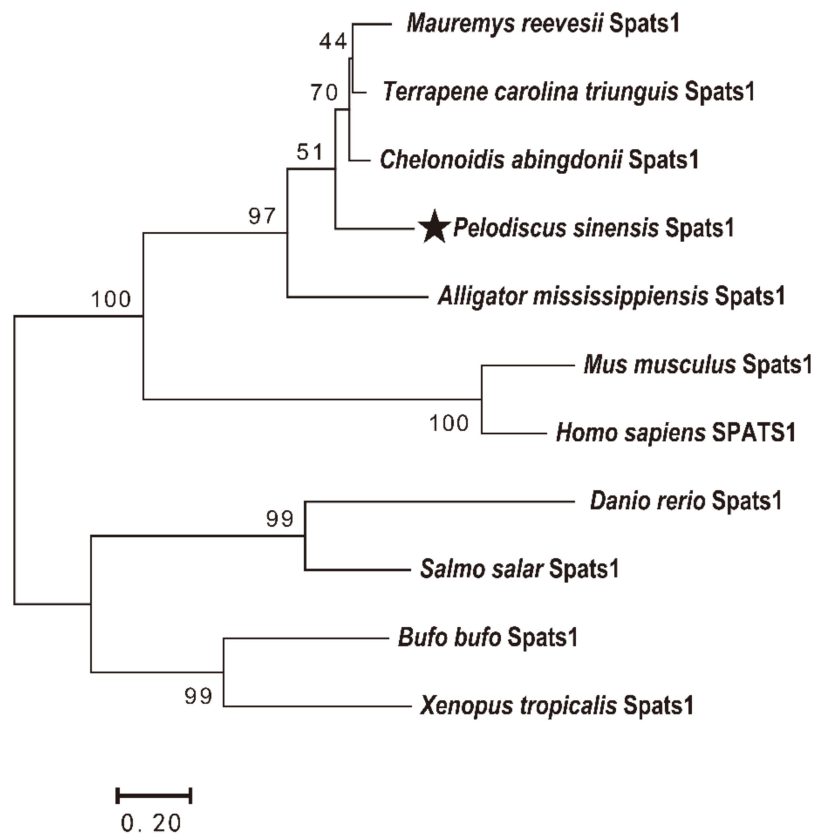

Figure 4. Phylogenetic tree constructed through the maximum likelihood method. The scale bar shows the genetic distance. The number at each node indicates the bootstrap value obtained for 1000 replicates.
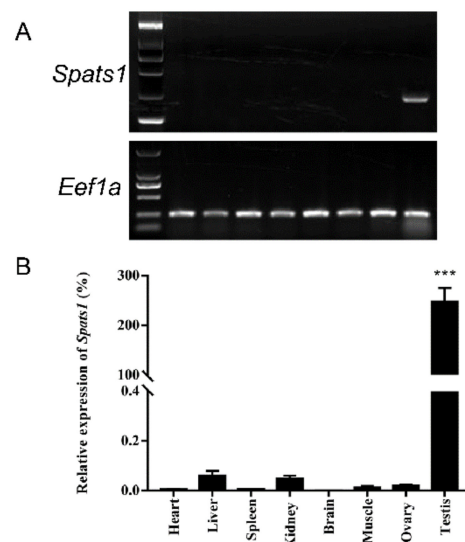

Figure 5. The distribution of *Spats1* mRNA in different tissues. (**A**) Tissue distribution gel electrophoresis map. (**B**) Tissue distribution assay results using qRT-PCR. *** $p < 0.001$.
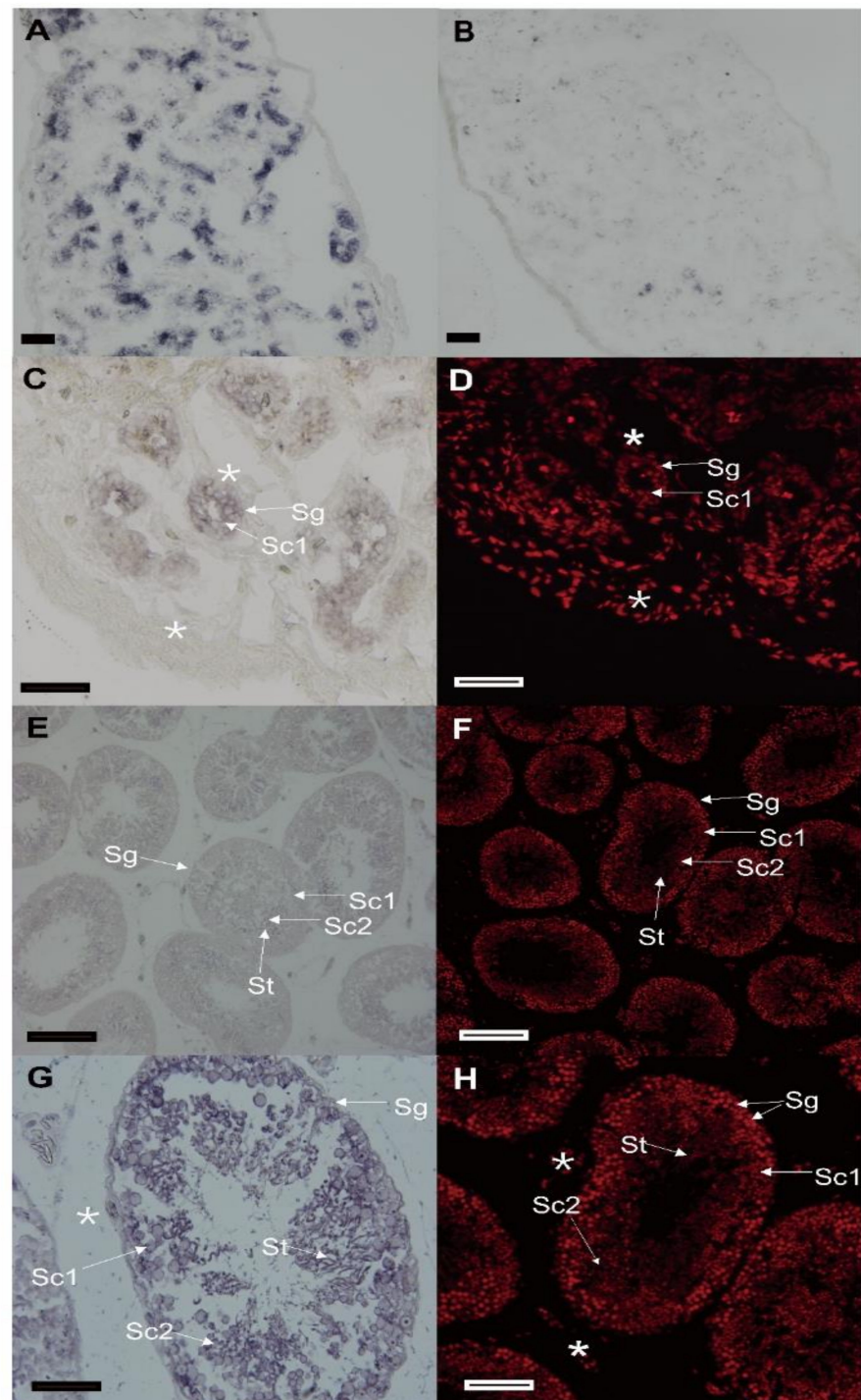
Figure 6. Localization of *Spats1* mRNA in testes by chemical in situ hybridization (CISH). The signals were developed by alkaline phosphatase (AP) staining (purple). *: somatic cells, St: spermatozoa, Sc1: primary spermatocytes, Sc2: secondary spermatocytes, Sg: spermatogonia. (**A**) Detected signal by antisense probe in testis section. (**B**) No signal was detected by sense probe in testis section. (**C,D**) The testis sections of one-year-old specimen; (**C**) Localization of *Spats1* in different cells; (**D**) Nucleus was stained with propidium iodide (PI). (**E,F**) The testis sections of a two-year-old specimen; (**E**) Localization of *Spats1* in different cells; (**F**) Nucleus was stained with PI. (**G,H**) The testis sections of a three-year-old specimen; (**G**) Localization of *Spats1* in different cells. H: Nucleus was stained with PI. Scale bars = 50 μm.

3.4. Expression Pattern of Spats1 mRNA in Testes of Different Seasons

In *P. sinensis*, spermatogenesis is accompanied by a distinct periodic rhythm. In this study, the expression pattern of the sex-bias gene *Spats1* in *P. sinensis* testes was studied. qRT-PCR was used to test the relative expression levels of *Spats1* mRNA in testes at different seasons. Overall, the expression of *Spats1* in male *P. sinensis* appeared to increase with age. The expression of *Spats1* was significantly lower in one-year-old males than in two- and three-year-old males in different seasons (DF = 24, F = 15.048, $p < 0.01$). Moreover, the expression of *Spats1* in one-year-old male was not significantly different between seasons. In two- and three-year-old males, the expression pattern of *Spats1* was significantly different across seasons (DF = 24, F = 15.048, $p < 0.01$). *Spats1* expression was lowest in April and highest in July (DF = 24, F = 15.048, $p < 0.01$). *Spats1* expression gradually decreased after July, but *Spats1* expression was still significantly higher in October than in April (DF = 24, F = 15.048, $p < 0.01$) (Figure 7).
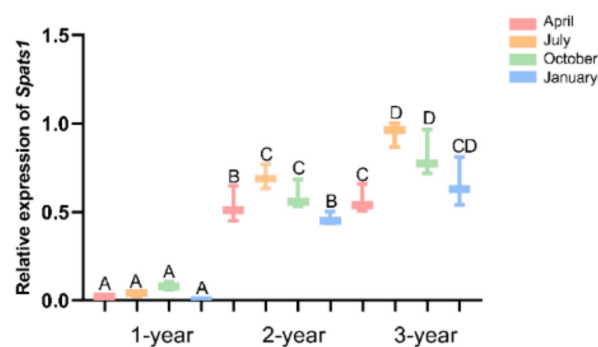

Figure 7. Expression of *Spats1* in testes of different ages in different seasons. Different letters represent the significance of differences between different groups ($p < 0.01$).

3.5. Effect of E2 and MT Treatment on Embryos

Compared to the untreated control, the tails of the newly hatched male and female *P. sinensis* turtles became more elongated after the E2 treatment (Figure 8A(b,e)). In contrast, the tails of MT-treated male and female *P. sinensis* became shorter and stouter (Figure 8A(c,f)). The qRT-PCR results showed that *Spats1* expression was decreased in embryonic testes after E2 treatment and up-regulated after MT treatment compared to the control group, but none of the differences were significant. In addition, neither E2 nor MT treatment had a significant effect on *Spats1* expression in female embryos (Figure 8B).

3.6. E2 and MT Treatment Were Performed on Adult Male and Female P. sinensis

E2 and MT treatments were performed on adult male and female *P. sinensis*, and it was found that both E2 and MT treatments could barely significantly affect *Spats1* expression in female. At 6 h, 12 h, 24 h, 48 h, and even 7 days after E2 and MT treatment, *Spats1* expression in females was always weak (Figure 9A). In contrast, in males, the expression of *Sptas1* in testes increased significantly after MT treatment, peaked at 6 h, and then began to gradually decrease. However, at 12 h, its expression level remained significantly higher than that of the untreated controls (DF = 14, F = 60.518, $p < 0.01$). 24 and 48 h after MT treatment, the expression level of *Spats1* in the testes continued to decrease, but was still significantly higher than the untreated controls (DF = 14, F = 60.518, $p < 0.05$). It was not until day 7 that the expression level of *Spats1* decreased to the same level as the initial expression. Interestingly, the expression of *Spats1* in the testes was almost unchanged after E2 treatment, either at 6 h, 12 h, 24 h, 48 h, or after 7 days (Figure 9B).

Figure 8. (**A**) E2- and MT-treated embryonic tails. (**a**) Female controls; (**b**) E2-treated females; (**c**) MT-treated females; (**d**) Male controls; (**e**) E2-treated males; (**f**) MT-treated males. (**B**) *Spats1* mRNA expression. Control-F: Control Female; Control-M: Control Male; E2-F: E2-treated females; E2-M: E2-treated males; MT-F: MT-treated females; MT-M: MT-treated males; Significant differences are represented by asterisks (* $p < 0.05$, ** $p < 0.01$).
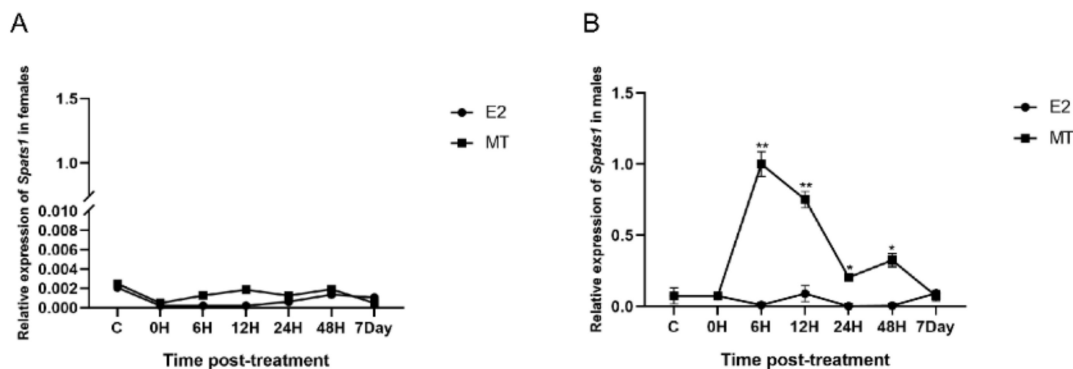

Figure 9. Relative expression of *Spats1* gene in adults after E2 and MT treatment. (**A**) Effects of E2 and MT on transcript abundance of *Spats1* gene in female adult *P. sinensis.* (**B**) Effects of E2 and MT on transcript abundance of *Spats1* gene in male adult *P. sinensis*. * means significant differences ($p < 0.05$); ** means significant differences ($p < 0.01$).

4. Discussion

In this study, the *Spats1* cDNA was cloned and characterized. An 849 bp cDNA fragment of *Spats1* encoding 283 amino acids was obtained. For protein 3D structures, although the N-terminal portion of Spats1 in *P. sinensis* begins with two α-helices, as in zebrafish and humans, the number of subsequent short α-helices is smaller than it is in either. The Spats1 3D structure in *P. sinensis* differs significantly from that of zebrafish and humans, suggesting that in *P. sinensis* may be evolutionarily more distant from humans and zebrafish. The results of protein 3D structures and multiple comparative analyses indicated the extensive homology of the *P. sinensis Spats1* gene with Testudinidae animals. This implies that the protein function of Spats1 in turtles may be different from that of other species.

Of the tissues examined in this study, *Spats1* mRNA expression was highest in the testis. *Spats1* has shown a similar expression profile in various tissues in mice [20]. Interestingly,

the distribution of *Spats1* in different tissues was almost consistent with the pattern of *Dmrt1* and anti-Müllerian hormone (Amh), which have been shown to be closely associated with testis differentiation in *P. sinensis* [15,31]. Similar to the gonadal structure of other vertebrates, the essential functional unit for sperm production in the testes of *P. sinensis* is the seminiferous tubule [32]. The arrangement of cells from the spermatogenic epithelium to the lumen is somatic cells, spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatids, and sperm [33–35]. To obtain insight into the function of *Spats1* in the development and differentiation of male germ cells, cellular localization of its transcripts in the testis was investigated. The obtained results revealed that *Spats1* mRNA was mainly located in spermatocytes, secondary spermatocytes, and spermatozoa. In rats, *Spats1* attains maximum levels during meiosis of the first spermatogenic wave, mostly in pachytene spermatocytes, whereas the signal was weaker in spermatogonia, Sertoli cells, and myoid cells [20]. Despite the fact that Spats1 is a testis-specific protein, the Spats1 loss-of-function mouse model analysis revealed that knockdown of Spats1 did not affect fertility in either males or females [36]. The testis-specific expression of *Spats1* and the expression pattern in different germ cells imply that *Spats1* may be closely associated with testicular differentiation and male germ cell differentiation in *P. sinensis*. In view of the differences in spermatogenesis mechanisms between reptiles and mammals, it remains to be investigated whether *Spats1* has the same effects on fertility in *P. sinensis* as in rats.

Seasonally breeding animals undergoes a periodic fluctuation in gonad development. In male reptiles, spermatogenesis and steroidogenesis have a highly seasonality [37–40]. In this study, *Spats1* expression in one-year-old males was very weak throughout the year, and *Spats1* expression was highest in the testes at three years old. Testosterone expression increased with age, and the peak of testosterone occurred at the peak of spermatogenic activity [41,42]. This implies that the differential expression of *Spats1* in different ages may be closely associated with the expression of testosterone. In addition, spermatogenesis of *P. sinensis* is initiated in the summer (June), and the mature spermatozoa are subsequently released from the epididymis in the autumn (October) [6,43]. In *P. sinensis*, the seminiferous epithelium is spermatogenically active in summer and autumn, but quiescent during the remaining part of the year [43]. The expression pattern of *Spats1* in this experiment implies that *Spats1* expression is seasonally influenced and closely related to spermatogenesis and release. Interestingly, a recent report on *P. sinensis* mentioned that the expression pattern of high-mobility group box 2 (Hmgb2) in the testes also strongly correlates with the season [44]. Therefore, we speculated that *Spats1* regulates sperm production and release and might be affected by effective accumulative temperature [45].

Gonadal development and sexual activity are not only determined by the interactions of multiple genes but also by environmental factors, such as temperature or hormone exposure [46,47], and exposure to exogenous hormones during gonadal differentiation can alter the population gender ratio [48,49]. Steroid hormones are natural and induce sex change processes [50], among which E2 is one of the most potent estrogens impacting sex differentiation [51] and MT is one of the most potent androgens impacting sex differentiation [52]. For instance, E2 induces sex reversal in *P. sinensis* and the protandrous barramundi (*Lates calcarifer*) [53,54], and MT controls secondary sexual characteristics in *Siniperca chuatsi* as well [55]. The same phenomenon has been confirmed in mammals and fish [56]. *P. sinensis* embryo is an excellent model for studying gonadal development and reproduction since development takes place in the egg, and we can intervene directly *in vitro*. In this experiment, both male and female *P. sinensis* embryonic tails changed significantly after E2 and MT treatments compared to the untreated group, indicating that the sexual characteristics of *P. sinensis* were affected by E2 and MT. The treatment of embryos with hormones resulted in significant alterations in the expression of many genes [57,58]. The expression of some sexually dimorphic genes is significantly altered in the embryo after sex hormone treatment in *P. sinensis*, such as *Dmrt1*, *Sox9* [15], and cytochrome p450 family 19 subfamily a (Cyp19a) [59]. However, in the present experiment, the expression of *Spats1* in embryos was not significantly altered after E2 and MT treatment. In contrast, in adults,

Spats1 expression was significantly increased in males after MT treatment, which is similar to the pattern in zebrafish, where the expression of gonadotrophin-releasing hormones 2 and 3 (*gnrh2* and *gnrh3*) is upregulated after MT treatment [57]. The expression pattern of *Spats1* in embryos and adults after sex hormone treatment suggests that *Spats1* responds to sex endosteroid hormone regulation only in adults, and it is hypothesized that the gene may not be involved in sex differentiation in *P. sinensis*. Androgenic stimulation results not only in the production of seminal fluid, but also promotes the maturation of sperms [60]. In this experiment, the significant elevation of *Spats1* expression after MT treated in adults, implying that *Spats1* may be positively regulated by MT, which in turn promotes sperm maturation and release in *P. sinensis*. Studies have shown that estrogen is important for sperm activity and that estrogen treatment affects the expression of sex-related genes [61,62]. Yet, the expression of *Spats1* in male *P. sinensis* changed weakly after E2 treatment in this experiment. This is different from the response pattern of other sexually dimorphic genes in adult *P. sinensis* after E2 and MT treatment, such as forkhead box L2 (*Foxl2*), whose expression was significantly altered in both males and females after either E2 or MT treatment [63]. This might be related to the concentration of estrogen treatment or that *Spats1* is not located in the estrogen regulatory signaling pathway in *P. sinensis*. As for the fact that *Spats1* expression in females has been very weak and almost unaffected by E2 and MT treatments, there are two possible reasons: inadequate concentration of hormone treatment, or the fact that *Spats1* is not involved in female physiological activities at all. Thus, the specific regulation mechanism involved in this process needs to be further studied.

5. Conclusions

In summary, *Spats1* is specifically expressed in the testis of *P. sinensis* with a seasonal expression pattern and is involved in spermatogenesis and release. Moreover, E2 and MT treatments revealed that *Spats1* only responded to MT treatment both in embryos and in adults. Thus, additional research is necessary to investigate the issue in the future. This study enriches the data on *Spats1* in *P. sinensis* and provides a reference for future studies on the molecular mechanisms of reproductive activity in this species and even other reptiles.

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Institutional Review Board Statement: The Animal Care and Ethics Committee of the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences (Guangzhou, China) approved this research (Approval Code: LAEC-PRFRI-20210148; Approval Date: 10 January 2021), and all experimental protocols and methods were performed in accordance with the relevant guidelines and regulations.

Informed Consent Statement: Not applicable.

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References

1. Yang, T.; Yang, W.-X. The dynamics and regulation of microfilament during spermatogenesis. *Gene* **2020**, *744*, 144635. [CrossRef] [PubMed]
2. Gribbins, K. Reptilian spermatogenesis: A histological and ultrastructural perspective. *Spermatogenesis* **2011**, *1*, 250–269. [CrossRef] [PubMed]
3. Rosati, L.; Agnese, M.; Lorenzo, M.; Barra, T.; Valiante, S.; Prisco, M. Spermatogenesis and regulatory factors in the wall lizard *Podarcis sicula*. *Gen. Comp. Endocrinol.* **2020**, *298*, 113579. [CrossRef]
4. Wang, Y.; Liu, X.; Li, W.; Zhao, J.; Liu, H.; Yu, L.; Zhu, X. Reproductive performance is associated with seasonal plasma reproductive hormone levels, steroidogenic enzymes and sex hormone receptor expression levels in cultured Asian yellow pond turtles (*Mauremys mutica*). *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **2021**, *254*, 110566. [CrossRef] [PubMed]
5. Resende, F.C.d.; Avelar, G.F.d. The sexual segment of the kidney of a tropical rattlesnake, *Crotalus durissus* (Reptilia, Squamata, Viperidae), and its relationship to seasonal testicular and androgen cycles. *J. Morphol.* **2021**, *282*, 1402–1414. [CrossRef] [PubMed]
6. Wang, P.; Li, X.; Wang, X.; Peng, N.; Luo, Z. Effects of dietary xanthophyll supplementation on growth performance, body color, carotenoids, and blood chemistry indices of chinese soft-shelled turtle *Pelodiscus sinensis*. *N. Am. J. Aquac.* **2020**, *82*, 394–404. [CrossRef]
7. Gauthier, J.; Cannatella, D.C.; De Queiroz, K.; Kluge, A.G.; Rowe, T. Tetrapod phylogeny. *Hierarchy Life* **1989**, *25*, 337–353.
8. Uno, Y.; Nishida, C.; Tarui, H.; Ishishita, S.; Takagi, C.; Nishimura, O.; Ishijima, J.; Ota, H.; Kosaka, A.; Matsubara, K.; et al. Inference of the protokaryotypes of amniotes and tetrapods and the evolutionary processes of microchromosomes from comparative gene mapping. *PLoS ONE* **2012**, *7*, e53027. [CrossRef]
9. Chen, H.; Huang, Y.; Liu, T.; Haseeb, A.; Ahmed, N.; Zhang, L.; Bian, X.; Chen, Q. Characteristics of seasonal spermatogenesis in the soft-shelled turtle. *Anim. Reprod. Sci.* **2020**, *214*, 106307. [CrossRef]
10. Zhang, L.; Han, X.; Li, M.; Bao, H.; Chen, Q. Spermiogenesis in soft-shelled turtle, *Pelodiscus sinensis*. *Anat. Rec. Adv. Integr. Anat. Evol. Biol.* **2007**, *290*, 1213–1222. [CrossRef]
11. Bian, X.; Zhang, L.; Yang, L.; Yang, P.; Ullah, S.; Zhang, Q.; Chen, Q. Ultrastructure of epididymal epithelium and its interaction with the sperm in the soft-shelled turtle *Pelodiscus sinensis*. *Micron* **2013**, *54*, 65–74. [CrossRef] [PubMed]
12. Viets, B.; Ewert, M.; Talent, L.; Nelson, C. Sex-determining mechanisms in squamate reptiles. *J. Exp. Zool.* **1994**, *270*, 45–56. [CrossRef]
13. İnanan, B.E.; Acar, Ü. Comparison of milt and blood parameters including testosterone and gonadotropins levels of sex-reversed female rainbow trout (*Oncorhynchus mykiss*) by different steroid hormones. *Aquac. Res.* **2021**, *52*, 1876–1884. [CrossRef]
14. Ruiz-García, A.; Roco, Á.S.; Bullejos, M. Sex differentiation in amphibians: Effect of temperature and its influence on sex reversal. *Sex. Dev.* **2021**, *15*, 157–167. [CrossRef] [PubMed]
15. Wang, L.; Hu, T.; Mi, M.; Yang, K.; Qian, G.; Ge, C. The effects of estrogen on gonadal differentiation and expressions of DMRT1 and SOX9 in *Pelodiscus sinensis*. *Acta Hydrobiol. Sin.* **2014**, *38*, 467–473.
16. Liang, H.; Meng, Y.; Cao, L.; Li, X.; Zou, G. Effect of exogenous hormones on R-spondin 1 (RSPO1) gene expression and embryo development in *Pelodiscus sinensis*. *Reprod. Fertil. Dev.* **2019**, *31*, 1425–1433. [CrossRef]
17. Kopania, E.; Larson, E.; Callahan, C.; Keeble, S.; Good, J. Molecular evolution across mouse spermatogenesis. *Mol. Biol. Evol.* **2022**, *39*, msac023. [CrossRef]
18. Geisinger, A.; Alsheimer, M.; Baier, A.; Benavente, R.; Wettstein, R. The mammalian gene pecanex 1 is differentially expressed during spermatogenesis. *Biochim. Et Biophys. Acta (BBA)-Gene Struct. Expr.* **2005**, *1728*, 34–43. [CrossRef]
19. Geisinger, A.; Dos Santos, A.; Benavente, R.; Wettstein, R. Identification and characterization of Srsp1, a rat gene differentially expressed during spermatogenesis and coding for a serine stretch-containing protein. *Cytogenet. Genome Res.* **2002**, *98*, 249–254. [CrossRef]
20. Capoano, C.A.; Wettstein, R.; Kun, A.; Geisinger, A. Spats 1 (Srsp1) is differentially expressed during testis development of the rat. *Gene Expr. Patterns* **2010**, *10*, 1–8. [CrossRef]
21. da Cruz, I.; Rodríguez-Casuriaga, R.; Santiñaque, F.F.; Farías, J.; Curti, G.; Capoano, C.A.; Folle, G.A.; Benavente, R.; Sotelo-Silveira, J.R.; Geisinger, A. Transcriptome analysis of highly purified mouse spermatogenic cell populations: Gene expression signatures switch from meiotic-to postmeiotic-related processes at pachytene stage. *BMC Genom.* **2016**, *17*, 294. [CrossRef] [PubMed]
22. Mouawad, N. SPATS1: New Molecular Target in Colorectal Cancer. Ph.D. Thesis, University of Pisa, Pisa, Italy, 2021.
23. Zhu, J.; Lei, L.; Chen, C.; Wang, Y.; Liu, X.; Geng, L.; Li, R.; Chen, H.; Hong, X.; Yu, L.; et al. Whole-Transcriptome Analysis Identifies Gender Dimorphic Expressions of Mrnas and Non-Coding Rnas in Chinese Soft-Shell Turtle (*Pelodiscus sinensis*). *Biology* **2022**, *11*, 834. [CrossRef]
24. Li, H.; Zhou, Z.; Wu, T.; Wu, Y.; Ji, X. Do fluctuations in incubation temperature affect hatchling quality in the Chinese soft-shelled turtle *Pelodiscus sinensis*? *Aquaculture* **2013**, *406*, 91–96. [CrossRef]
25. Du, W.G.; Ji, X. The effects of incubation thermal environments on size, locomotor performance and early growth of hatchling soft-shelled turtles, *Pelodiscus sinensis*. *J. Therm. Biol.* **2003**, *28*, 279–286. [CrossRef]
26. Zhang, Y.; Xiao, L.; Sun, W.; Li, P.; Zhou, Y.; Qian, G.; Ge, C. Knockdown of R-spondin1 leads to partial sex reversal in genetic female Chinese soft-shelled turtle *Pelodiscus sinensis*. *Gen. Comp. Endocrinol.* **2021**, *309*, 113788. [CrossRef]

27. Zhang, Y.; Xiao, L.; Sun, W.; Li, P.; Zhou, Y.; Qian, G.; Ge, C. Characterization of the *Pelodiscus sinensis* polymeric immunoglobulin receptor (*P. sinensis* pIgR) and its response to LPS and *Aeromonas sobria*. *Dev. Comp. Immunol.* **2021**, *121*, 104072.

28. Cai, H.; Sun, W.; Liu, H.; Bao, H.; Zhang, H.; Qian, G.; Ge, C. Molecular cloning and expression pattern of Amh and its preliminary functional analysis in male sexual differentiation in *Pelodiscus sinensis*. *Sci. Sin. Vitae* **2016**, *46*, 1423–1433.

29. Tokita, M.; Kuratani, S. Normal embryonic stages of the Chinese softshelled turtle *Pelodiscus sinensis* (Trionychidae). *Zool. Sci.* **2001**, *18*, 705–715. [CrossRef]

30. Li, W.; Zhu, X.; Xu, H.; Zhao, J.; Chen, C. A PCR Amplification Primer, Method and Kit for Rapid Identification of Genetic Traits in the Chinese Soft-shelled Turtle. China Patent CN108841945B, 5 May 2020.

31. Sun, W.; Cai, H.; Zhang, G.; Zhang, H.; Bao, H.; Wang, L.; Ye, J.; Qian, G.; Ge, C. Dmrt1 is required for primary male sexual differentiation in Chinese soft-shelled turtle *Pelodiscus sinensis*. *Sci. Rep.* **2017**, *7*, 4433. [CrossRef]

32. Cheng, C.Y.; Mruk, D.D. The biology of spermatogenesis: The past, present and future. *Philos. Trans. R Soc. Lond. B Biol. Sci.* **2020**, *365*, 1459–1463. [CrossRef]

33. Sharma, R.; Agarwal, A. Spermatogenesis: An overview. *Sperm Chromatin* **2011**, 19–44. [CrossRef]

34. Johnson, L. Efficiency of spermatogenesis. *Microsc. Res. Technol.* **1995**, *32*, 385–422. [CrossRef] [PubMed]

35. Saunders, P. Germ cell-somatic cell interactions during spermatogenesis. *Reprod. Suppl.* **2003**, *61*, 91–101. [CrossRef] [PubMed]

36. Capoano, C.A.; Ortiz-Laquintana, L.A.; Rodríguez-Casuriaga, R.; Schlapp, G.; Meikle, M.N.; Mulet, A.P.; Crispo, M.; Benavente, R.; Geisinger, A. SPATS1 (spermatogenesis-associated, serine-rich 1) is not essential for spermatogenesis and fertility in mouse. *PLoS ONE* **2021**, *16*, e0251028. [CrossRef]

37. Jiménez, R.; Burgos, M.; Barrionuevo, F.J. Circannual testis changes in seasonally breeding mammals. *Sex. Dev.* **2015**, *9*, 205–215. [CrossRef]

38. Mahmoud, I.; Licht, P. Seasonal changes in gonadal activity and the effects of stress on reproductive hormones in the common snapping turtle, *Chelydra serpentina*. *Gen. Comp. Endocrinol.* **1997**, *107*, 359–372. [CrossRef]

39. Ribeiro, Y.M.; de Matos, S.A.; Domingos, F.F.T.; Dos Santos, H.B.; Vieira, A.B.C.; Bazzoli, N.; Rizzo, E. Germ cell proliferation and apoptosis during testicular regression in a seasonal breeding fish kept in captivity. *Tissue Cell* **2017**, *49*, 664–671. [CrossRef]

40. Lofts, B. Patterns of spermatogenesis and steroidogenesis in male reptiles. In *Reproduction and Evolution*; Calaby, J.H., Tyndale-Boscoe, C.H., Eds.; Australian Academic Science: Canberra, Australia, 1977; pp. 127–136.

41. Radder, R.; Shanbhag, B.; Saidapur, S. Pattern of plasma sex steroid hormone levels during reproductive cycles of male and female tropical lizard, *Calotes versicolor*. *Gen. Comp. Endocrinol.* **2001**, *124*, 285–292. [CrossRef]

42. Yu, W.; Li, H.; Zhang, G.; Kang, X.; Li, Q.; Cao, J.; Xu, E. Serum sex hormonal levels and gonadal development in broodstock soft-shelled turtle *Trionyx sinensis* in a green house. *Fish. Sci.* **2012**, *31*, 264–269.

43. Zhang, L.; Han, X.K.; Qi, Y.Y.; Liu, Y.; Chen, Q.S. Seasonal effects on apoptosis and proliferation of germ cells in the testes of the Chinese soft-shelled turtle, *Pelodiscus sinensis*. *Theriogenology* **2008**, *69*, 1148–1158. [CrossRef]

44. Li, W.; Zhu, J.; Lei, L.; Chen, C.; Liu, X.; Wang, Y.; Hong, X.; Xu, H.; Zhu, X. The Seasonal and Stage-Specific Expression Patterns of HMGB2 Suggest Its Key Role in Spermatogenesis in the Chinese Soft-Shelled Turtle (*Pelodiscus sinensis*). *Biochem. Genet.* **2022**, 1–14. [CrossRef] [PubMed]

45. Sukhan, Z.P.; Cho, Y.; Sharker, M.R.; Hossen, S.; Rha, S.J.; Kho, K.H. Effective accumulative temperature affects gonadal maturation by controlling expression of GnRH, GnRH receptor, serotonin receptor and APGWamide gene in Pacific abalone, *Haliotis discus hannai* during broodstock conditioning in hatcheries. *J. Therm. Biol.* **2021**, *100*, 103037. [CrossRef] [PubMed]

46. Breitenbach, A.T.; Bowden, R.M.; Paitz, R.T. Effects of Constant and Fluctuating Temperatures on Gene Expression During Gonadal Development. *Integr. Comp. Biol.* **2022**, icac011. [CrossRef] [PubMed]

47. Di Lorenzo, M.; Mileo, A.; Laforgia, V.; De Falco, M.; Rosati, L. Alkyphenol Exposure Alters Steroidogenesis in Male Lizard *Podarcis siculus*. *Animals* **2021**, *11*, 1003. [CrossRef] [PubMed]

48. Othman, R.; Ron, X.J.; Yao, H.; O'Bryant, P.; Rapp, D.; Pei, J.C.; Pei, J.C.; Wang, H.P. The effect of methyltestosterone (MT) on sex differentiation and growth in juvenile yellow perch (*Perca flavescens*). *Fish Physiol. Biochem.* **2022**, *48*, 161–171. [CrossRef]

49. Cai, Y.; He, Z.; Zhang, Q.; Wang, X.; Zhang, S.; He, L.; Yang, D.; Zhang, M.; Yan, T. Oestradiol upregulates the expression of Cyp19a1a and the feminization ratio in *Schizothorax prenanti*. *Aquac. Rep.* **2022**, *24*, 101138. [CrossRef]

50. Gardner, L.; Anderson, T.; Place, A.R.; Dixon, B.; Elizur, A. Sex change strategy and the aromatase genes. *J. Steroid Biochem. Mol. Biol.* **2005**, *94*, 395–404. [CrossRef]

51. Jiang, Y.; Luo, H.; Hou, M.; Chen, J.; Tao, B.; Zhu, Z.; Song, Y.; Hu, W. Aromatase inhibitor induces sex reversal in the protogynous hermaphroditic rice field eel (*Monopterus albus*). *Aquaculture* **2022**, *551*, 737960. [CrossRef]

52. Zhu, Q.; Han, C.; Liu, S.; Ouyang, H.; Liu, D.; Zhang, Z.; Huang, J.; Han, L.; Li, S.; Li, G. Development and gene expression analysis of gonad during 17α-methyltestosterone-induced sex reversal in mandarin fish (*Siniperca chuatsi*). *Aquac. Rep.* **2022**, *23*, 101049. [CrossRef]

53. Li, P.; Guo, Y.; Jin, L.; Liang, X.; Chen, G.; Sun, W.; Xiao, L.; Qian, G.; Ge, C. ESR1 mediates estrogen-induced feminization of genetic male Chinese soft-shelled turtle. *Biol. Reprod.* **2022**, ioac088. [CrossRef]

54. Banh, Q.Q.; Guppy, J.L.; Domingos, J.A.; Budd, A.M.; Pinto, R.C.; Marc, A.F.; Jerry, D.R. Induction of precocious females in the protandrous barramundi (*Lates calcarifer*) through implants containing 17β-estradiol-effects on gonadal morphology, gene expression and DNA methylation of key sex genes. *Aquaculture* **2021**, *539*, 736601. [CrossRef]

55. Weber, G.M.; Leeds, T.D.; Schneider, R.P. Sex reversal of female rainbow trout by immersion in 17α-methyltestosterone. *Aquaculture* **2020**, *528*, 735535. [CrossRef]

56. Delbes, G.; Blázquez, M.; Fernandino, J.I.; Grigorova, P.; Hales, B.F.; Metcalfe, C.; Navarro-Martín, L.; Parent, L.; Robaire, B.; Rwigemera, A.; et al. Effects of endocrine disrupting chemicals on gonad development: Mechanistic insights from fish and mammals. *Environ. Res.* **2022**, *204*, 112040. [CrossRef] [PubMed]

57. Spaan, K.; Haigis, A.C.; Weiss, J.; Legradi, J. Effects of 25 thyroid hormone disruptors on zebrafish embryos: A literature review of potential biomarkers. *Sci. Total Environ.* **2019**, *656*, 1238–1249. [CrossRef]

58. Darras, V.M. The role of maternal thyroid hormones in avian embryonic development. *Front. Endocrinol.* **2019**, *10*, 66. [CrossRef]

59. Liang, H.W.; Meng, Y.; Cao, L.H.; Li, X.; Zou, G.W. Expression and characterization of the cyp19a gene and its responses to estradiol/letrozole exposure in Chinese soft-shelled turtle (*Pelodiscus sinensis*). *Mol. Reprod. Dev.* **2019**, *86*, 480–490. [CrossRef]

60. Dzyuba, V.; Cosson, J.; Papadaki, M.; Mylonas, C.C.; Steinbach, C.; Rodina, M.; Tučkova, V.; Linhart, O.; Shelton, W.; Gela, G. Influence of Environmental Temperature and Hormonal Stimulation on the In Vitro Sperm Maturation in *Sterlet Acipenser ruthenus* in Advance of the Spawning Season. *Animals* **2021**, *11*, 1417. [CrossRef]

61. Dostalova, P.; Zatecka, E.; Dvorakova-Hortova, K. Of oestrogens and sperm: A review of the roles of oestrogens and oestrogen receptors in male reproduction. *Int. J. Mol. Sci.* **2017**, *18*, 904. [CrossRef]

62. Komrsková, K. The Physiology and Reproductive Potential of Sperm. Ph.D. Thesis, Charles University, Prague, Czech Republic, 2021.

63. Gao, L.L.; Diao, X.M.; Li, Y.; Zhai, X.L.; Zhou, C.L. Molecular cloning and expression of foxl2 gene induced by exogenous hormone in the *pelodiscus sinensis*. *Acta Hydrobiol. Sin.* **2019**, *43*, 7.