



New insights into the role of myostatin in fish fertility based on the findings in *mstnb*-deficient Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Myostatin (Mstn) was reported to be involved in multiple biological processes including the regulation of skeletal muscle growth, lipid metabolism and reproduction in vertebrates, therefore, it might play an essential role in the fine balance between growth and reproduction. In order to clarify the roles of Mstn in fish reproduction, we systematically investigated the gonadal development and fertility of *mstnb* mutants in Nile tilapia (*Oreochromis niloticus*). In the present study, *mstnb* was found to be expressed in the different cell types of gonads by fluorescence *in situ* hybridization (FISH). Mutation of *mstnb* resulted in excessive proliferation of phase II follicles in the ovaries while the numbers of phase III follicles were significantly reduced at 210 days after hatching (dah). Interestingly, significant decline of *cyp19a1a* gene expression, the serum 17 β -estradiol (E2), vitellogenin (VTG) were detected at 210 dah. At 360 dah, the ovarian development of *mstnb*^{-/-} XX tilapia was partially recovered and female fish spawn regularly, however, they were subfertile and produced the poor-quality eggs with significantly lower offspring survival rate. The dramatic up-regulation of *cyp19a1a* gene expression and serum E2 level at 360 dah might account for the recovery of ovarian development. In *mstnb*^{-/-} males, excessive proliferation of spermatogonia and retardation of testicular differentiation were found throughout the whole development stages. In *mstnb*^{-/-} males, evident declines of gonadosomatic index (GSI), *cyp11b2* gene expression, and 11-Ketotestosterone (11-KT) were detected at 210 dah, while the mRNA level of *cyp11b2* and serum 11-KT level were significantly upregulated at 360 dah. However, *mstnb*^{-/-} males were infertile since mutation of *mstnb* gene led to excessive increase of apoptosis of testicular cells and spermiation obstacle. Taken together, these results indicated that Mstn might be an essential regulator for the maintenance of fish fertility.

1. Introduction

The assumption of a trade-off between body growth and fecundity is a common feature of life history models (Roff, 2000). In aquaculture, sterile strains were preferred by exhibiting several commercial advantages including increased growth performance, improved flesh quality, disease resistance, increased environmental tolerance and elimination the risk of spreading modified genetic material to wild fish populations (Bartley et al., 2000; Hallerman and Kapuscinski, 1995; Rahman et al.,

2005; UmEKalsoom et al., 2009). Optimized fertility and growth were critically regulated by interplay and coordination of multiple networks involving various factors, such as steroids, transcription factors and growth factors (Hull and Harvey, 2014; Yan et al., 2020; Zhou et al., 2021). Understanding the interactions and mechanisms of these networks is important for development of new strains with the advantages of higher economics efficiency and environmental sustainability.

Myostatin (MSTN), also known as growth differentiation factor 8 (GDF8), is a well-known negative regulator of muscle growth and a

Abbreviations: MSTN/Mstn, myostatin; 11-KT, 11-ketotestosterone; E2, 17 β -estradiol; VTG, vitellogenin; Cyp11b2, cytochrome P450, family 11, subfamily B, polypeptide 2; Cyp19a1a aromatase, cytochrome P450, family 19, subfamily A, polypeptide 1a; dah, days after hatching; qRT-PCR, quantitative reverse transcription polymerase chain reaction; EIA, enzyme immunoassay; GSI, gonadosomatic index; IF, Immunofluorescence; FISH, Fluorescence in situ hybridization; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labeling.

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member of the transforming growth factor-beta (TGF β) family. So far, *mstn* homozygous mutants, which displayed “double-muscling” phenotype and fast growth rate, have been successively established in farmed species in vertebrates (Li et al., 2016; Qian et al., 2015b; Zhao et al., 2022). Intriguingly, increasing evidences revealed that MSTN/Mstn might be also involved in the regulation of ovarian function. Previous study showed that *MSTN* is expressed in granulosa cells and follicular fluid in human beings (Chang et al., 2015b). The most notably expression of *Mstn* in chicken gonads during embryo stages (Kubota et al., 2007) and various-sized antral follicles in the ovaries of bovine (Skinner et al., 2008) were also documented. Another study showed that that *MSTN* increased 17 β -estradiol production in granulosa cells by increasing cytochrome P450 aromatase (*cyp19a1a*, aromatase) and FSH receptor expression levels (Chang et al., 2016a). Previous research also found that *MSTN* played an essential role in modulating granulosa and thecal steroidogenesis (Cheewasopit et al., 2018). These results indicated that *MSTN*, being an intraovarian factor, may play a critical role in female ovarian development and steroid synthesis. However, the effects of *Mstn* in testes development and male fertility remains to be controversial in vertebrates. Vaughan et al. (2020) found that inhibition of Activin/Myostatin signaling induced muscle hypertrophy, and smaller testes in mice. And the testes phenotype persists longer than muscle hypertrophy following the withdrawal of sActRIIB (an Activin/Myostatin ligand trap) treatment (Vaughan et al., 2020). Previous studies revealed that two *mstn* genes, known as *mstna* and *mstnb*, had been identified in several examined teleosts, with significant expression in numerous tissues notably in muscle and gonads (Helterline et al., 2007). Previous work revealed that deficiency of *mstn* gene contribute to the over-growth and over-proliferation of muscle cells in teleosts including red sea bream (*Pagrus major*), yellow catfish (*Pelteobagrus fulvidraco* Richardson) and so on (Ohama et al., 2020; Wu et al., 2023; Zhang et al., 2020). These data indicated the importance of *Mstn* in skeletal muscle growth and development in vertebrates. However, the involvements of *Mstn* in teleost reproduction, particularly in gonad development and fertility, remain unknown. Therefore, further investigations are required to clarify the roles of *Mstn* in fish fertility.

Nile tilapia is a worldwide farmed fish with an XX-XY sex-determination system. The availabilities of genetic all-XX and all-XY fish (Sun et al., 2014), shorter spawning cycle (14 days), and high-quality genome sequences (Brawand et al., 2014) have made it an excellent model for the study of gene expression and function in relation to reproduction and fertility. Particularly, the *mstnb*^{-/-} tilapia, which displayed fast growth and increased skeletal muscle mass, have been established in our previous work (Wu et al., 2023). In the present study, we analyzed the impacts of *mstnb* gene mutation on gonad phenotype, germ cell number, gonadal gene expression and serum E2 (17 β -estradiol)/11-KT

(11-ketotestosterone) level. Subsequently, we evaluated the fertility of *mstnb*^{-/-} fish by detecting the spawning frequency, offspring survival rate, and urogenital papilla histology. To our knowledge, our work, for the first time, shed light on the specific role of *Mstn* in teleost gonadal development and fertility.

2. Materials and method

2.1. Animals

The Nile tilapia was reared in recirculating aerated freshwater tanks at a temperature of 26 $\pm$ 2 $^{\circ}$ C, under a natural photoperiod. Fertility assessment was carried out using the *mstnb* mutants generated in our previous study (Wu et al., 2023). To identify the genetic sex of both wild-type (WT) and *mstnb*^{-/-} Nile tilapia, PCR amplification was performed (Fig. S1). For this purpose, a pair of sex-specific primers (SPF/SPR) listed in Table 1 were employed (Li et al., 2015; Sun et al., 2014). All animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University (No. IACUC-20181015–12, 15 October 2018).

2.2. Sampling and histological analysis

To investigate the roles of *Mstnb* in gonadal development, the gross morphology and histology of WT and *mstnb*^{-/-} ovaries and testes were analyzed at 210 and 360 days after hatching (dah). The fish were anesthetized with MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and the gonad morphology of the mutants and WT fish was imaged by stereomicroscope (Leica, Bensheim, Germany) after biopsy. The body and gonad weight were measured for gonadosomatic index (GSI) calculating ($n = 4$ for each genotype). Then, the gonads were fixed in Bouin's solution for 24 h at room temperature. The fixed samples were then processed as follows: serial dehydration in 70%, 80% and 90% ethanol for 1.5 h each, 95% ethanol for 2 h and 100% ethanol three times for 1 h each; sequential clearance in xylene and ethanol mixture (1:1) for 30 min and xylene twice for 20–120 min each (The immersion time varies for different tissues); and infiltration in paraffin 2 h. The samples were sectioned at 5–7 μ m thickness using the Leica microtome (Leica Microsystems, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (H&E) as described previously (Wang et al., 2007). Photographs were taken under Olympus BX51 light microscope (Olympus, Tokyo, Japan).

Table 1
Primers used in the present study.

Primer name	Primer sequence (5' to 3')	Gene ID	Purpose
SPF	ATGGCTCCGAGACCTTGACTG	ENSONIG00000004781	Identification of genetic sex
SPR	CAGAAATGTAGACGCCAGGTAT		
<i>mstnb</i> -ISH-F	TAGACACGGATCAATGCGCT	ENSONIG00000007163	FISH
<i>mstnb</i> -ISH-R	CTCTGCGAAGGTCACAGCTA		
<i>mstna</i> -ISH-F	CCACAGCATCAAGTCGCAAA	ENSONIG000000012192	
<i>mstna</i> -ISH-R	GCGTGTCTCCACAGACTCTT		
M13F	CGCCAGGGTTTCCAGTCACG	Sequencing Primers	
M13R	AGCGGATAACAATTTACACAG		
β -actin-Q-F	GGCATCACACCTTCTACAACGA	ENSONIG00000003145	qRT-PCR
β -actin-Q-R	ACGCTCTGTGAGGATCTTCA		
<i>cyp19a1a</i> -Q-F	TGTGCCAGGTCCTTCTTTCT	ENSONIG00000000155	
<i>cyp19a1a</i> -Q-R	CACATGGTGCACTGCTGAAG		
<i>cyp11b2</i> -Q-F	AAAGAAGTCCTCAGGTTGTACC	ENSONIG000000006462	
<i>cyp11b2</i> -Q-R	GACCAAAGTTCACGAGGTATG		
<i>mstna</i> -Q-F	CGCACAAATCCAATCGCCC	ENSONIG000000012192	
<i>mstna</i> -Q-R	CCAGCGTCGGTGTCAATCTT		

F, forward primer; R, reverse primer; FISH, Fluorescence in situ hybridization; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

2.3. Immunofluorescence (IF), fluorescence in situ hybridization (FISH) and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL)

FISH experiments were performed to examine the gene expression of *mstnb* in WT fish. The samples were fixed in 4% paraformaldehyde in $0.85 \times$ PBS at 4 °C overnight. Specimens were embedded in paraffin and sectioned at 5 μ m. FISH was performed to examine the gene expression as described previously (Li et al., 2020). Fragments of partial *mstnb* and *mstna* ORF was amplified and cloned into pGEM-T easy vector via TA cloning. Probes for *mstnb* and *mstna* antisense or sense digoxigenin-labeled RNA strands were transcribed *in vitro* from a linearized pGEM-T easy-*mstnb*/*mstna* plasmid using the T7 RNA labeling kit (Roche, Mannheim, Germany). For more sensitive FISH detection, the tyramide signal amplification (TSATM) Plus Fluorescence Systems (Akoya Biosciences, Marlborough, MA, USA) were used according to the manufacturer's instructions. IF analysis was conducted using the same methods employed for immunohistochemistry (IHC) experiments (Li et al., 2012). Goat anti-rabbit polyclonal antibodies of cytochrome P450, family 19, subfamily A, polypeptide 1a (Cyp19a1a, AB_2629226) and cytochrome P450, family 11, subfamily B, polypeptide 2 (Cyp11b2, AB_2650466) were diluted at 1:1000. As a negative control, the primary antibody was replaced with normal rabbit serum. The slides were then incubated with Alexa Fluor 488 or 594 conjugated secondary antibody (Invitrogen, Shanghai, China) at a 1:2000 dilution in blocking solution. The nuclei were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Carlsbad, CA, USA).

Apoptosis of testicular cells was evaluated by staining paraffin sections of the testes of WT and *mstnb*^{-/-} XY fish at 210 and 360 dah with in situ cell death detection kit, One Step TUNEL Apoptosis Assay Kit (Red Fluorescence) (Beyotime, Shanghai, China) according to the manufacturer's protocol. Apoptotic positive signaling in the testes (entire median section, n = 3 for each genotype) were detected by image J software for statistical analysis. All the images for IF, FISH and apoptosis observation were captured using an FV3000 confocal microscope (Olympus, Tokyo, Japan).

2.4. Follicle and spermatogonia counting

Fixed ovaries and testes were embedded in paraffin and sectioned at 5–7 μ m thickness. The ovaries and testes from 4 fish of each genotype (WT XX fish, *mstnb*^{-/-} XX fish, WT XY fish and *mstnb*^{-/-} XY fish) were dissected at 210 and 360 dah, stained with H&E and used for follicles and spermatogonia cell counting. All sections were imaged using an Olympus BX51 optical microscope (Olympus, Tokyo, Japan). The numbers of oocytes at different developmental stages (phase I, II, III, IV) and spermatogonia in each section were quantified manually according to the criteria described previously (Coward and Bromage, 1998; Neves et al., 2009).

2.5. Measurement of serum E2, vitellogenin (VTG) and 11-KT level

Blood samples were collected from 4 fish of each groups (WT XX fish, *mstnb*^{-/-} XX fish, WT XY fish and *mstnb*^{-/-} XY fish) at 210 and 360 dah, then kept at 4 °C overnight. The serum (n = 4) was collected after centrifugation (3000 rpm, 20 min) and stored at -80 °C until use. Serum E2 (AB_2832924), 11-KT (AB_328061), VTG (MM_3367502) concentrations were measured using an EIA (enzyme-linked immunosorbent assay) kit (Cayman, Michigan, USA) and Fish VTG ELISA kit (Meimian, Jiangsu, China) according to the manufacturer's instructions.

For serum E2/11-KT detection, the approach had been previously used to quantify serum hormone levels in tilapia. In brief, a 96-well plate with two blanks, two nonspecific binding wells, two maximum binding wells and an eight-point standard curve (in duplicate) was analyzed to ensure accurate results. A standard curve ranging from 0.78 to 100 pg/ml was constructed with two-fold dilutions of a 1 ng/ml bulk standard.

Each serum sample from WT XX, *mstnb*^{-/-} XX, WT XY, and *mstnb*^{-/-} XY fish was measured in two dilutions and each dilution was assayed in duplicate. All necessary reagents including buffer, standard, serum samples, tracer, and antiserum, were sequentially loaded into the corresponding wells according to the manufacturer's instructions. The plate was then incubated at 4 °C for 18 h to increase the reaction sensitivity. All plates underwent an additional rinse with wash buffer and the plate underwent a 60–90 min re-incubation with Ellman's reagent in the dark. The absorbance was measured at a 405 nm wavelength using a Multiskan GO microplate reader (Thermo Fisher Scientific, USA). The binding ratio of E2/11-KT and the E2/11-KT tracer versus the E2/11-KT concentration were plotted using linear (y) and log (x) scales and a 4-parameter logistic fit was performed. Based on the binding ratio of each well, the E2/11-KT sample concentrations (pg/ml) were determined using the fitting formula calculated from the standard curve.

For the detection of serum VTG, the protocol was conducted as follows. Initially, a dilution series of the original density standard was prepared by diluting a 480 μ g/L bulk standard with two-fold dilutions, ranging from 30 to 480 μ g/L. Subsequently, a 48-well plate was employed, containing two blanks and a five-point standard curve (in duplicate), to ensure precise outcomes. Two dilutions of each serum sample from WT XX, *mstnb*^{-/-} XX, WT XY, and *mstnb*^{-/-} XY fish were measured, and each dilution was assayed in duplicate. All necessary reagents, including sample dilution, standard, serum samples, wash solution, HRP-conjugate reagent, chromogen solution, and stop solution, were loaded sequentially into their respective wells, following the manufacturer's instructions. The plate was then incubated at 37 °C for 30 min to enhance reaction sensitivity. Subsequently, all plates were rinsed with wash solution and underwent a 30-minute re-incubation with HRP-conjugate reagent. After another round of washing, the plate was incubated for 10 min in the dark with chromogen solution. Absorbance at 450 nm was then measured immediately after adding stop solution, and within 15 min. Alternatively, the straight-line regression equation of the standard curve, formed by the standard density and the OD value, was used to calculate the sample density by substituting the sample OD value into the equation. The resulting density was then multiplied by the dilution factor to obtain the actual sample density.

2.6. Fertility analyses

Spawning frequency, offspring survival rate, and urogenital papilla histology were used for fertility analyses. Eight female fish with four fish of both WT and *mstnb*^{-/-} XX fish at 360 dah were used for a 60-day spawning frequency detection assay after they spawned once naturally. To check the fertility, 300 eggs from both WT and *mstnb*^{-/-} XX fish were used for the artificial insemination with excessive amount of semen (~500 μ L) from the same WT male fish. In total, three different control male fish were paired with WT (n = 4) or *mstnb*^{-/-} XX (n = 4) fish, respectively. Moreover, the survival rate of the offspring was calculated from 1 to 10 days after fertilization.

At 360 dah, the semen of WT XY fish (n = 3) were collected by manual extrusion, while the semen of *mstnb*^{-/-} XY fish (n = 3) were unable to be squeezed out. Further urogenital papilla observation demonstrated that urogenital tract samples from three WT XY fish and three *mstnb*^{-/-} XY fish were serially sectioned and used for H&E staining to evaluate the urogenital papilla and its external smooth muscle distribution. The thickness of smooth muscle in the urogenital papilla of WT and *mstnb*^{-/-} XY fish were determined using image J software.

2.7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Ovaries and testes of the WT and *mstnb*^{-/-} fish were dissected at 210 and 360 dah to check the effects of *mstnb* gene mutation on gonadal gene expression. Total RNA (1 μ g) was extracted and reverse transcribed using PrimeScript RT Master Mix Perfect Real Time Kit according to the

manufacturer's instructions (Takara, Dalian, China). qRT-PCR was performed on an ABI7500 qRT-PCR machine (Thermo Fisher, Waltham, MA, USA), according to the protocol of SYBR1 Premix Ex Taq™ II (Takara, Dalian, China). The relative abundance of key genes in different sample was evaluated using the formula $R = 2^{-\Delta\Delta C_t}$. The reference gene *tilapia β -actin* was used to normalize the expression values.

Primers for above experiments including *FISH* and qRT-PCR were listed at Table 1.

2.8. Statistical Analysis

All data were presented as the mean $\pm$ standard deviation (mean $\pm$ SD). Two-tailed unpaired Student's t-test was used to determine statistically significant difference between two groups. One-way ANOVA was performed for comparisons followed by Tukey's test for multiple comparison test. Statistics analyses were performed using GraphPad Prism 8 software package (GraphPad Software, La Jolla, USA). In all analyses, $P < 0.05$ was considered to be significantly different.

3. Results

3.1. Cellular localization of *mstnb* in tilapia ovary and testis

qRT-PCR analyses revealed that *mstnb* mRNA was detected in the ovaries and testes from 20 dah to 180 dah. The expression level in the ovaries peaked at 60 dah, and then decreased and maintained a low expression level (Fig. 1A). In the testes, the expression of *mstnb* was higher at 60 and 90 dah than that of other stages (Fig. 1B). By *FISH* assay, *mstnb* was detected in the cytoplasm of oocytes at phase I-II, follicular cells of late-phase oocytes in the ovary (Fig. 1C a). It was mainly localized in the epithelial lining of vas deferens and Leydig cells

in testes (Fig. 1C b-c) at 180 dah.

3.2. Homozygous mutation of *mstnb* led to the retardation of ovarian development

To investigate the roles of *Mstn* in ovarian development, the morphology and histology of the gonad in the WT and *mstnb*^{-/-} XX fish were analyzed. Morphological analysis showed that the ovarian development of *mstnb*^{-/-} XX fish were similar to that of WT XX fish at both 210 and 360 dah (Fig. 2A-B, E-F), which was confirmed by the undifferentiated GSI between two groups (Fig. 2I-J). Further histological analysis indicated that excessive proliferation of phase II follicles and few phase III follicles were observed in the *mstnb*^{-/-} ovaries at 210 dah, while the number of follicles from phase I to IV observed in the *mstnb*^{-/-} ovaries was similar to that of the WT ovaries at 360 dah (Fig. 2C-D, G-H, K-L). These results revealed that the ovarian development was delayed at 210 dah and the development caught up later by 360 dah in *mstnb*^{-/-} female fish.

3.3. Mutation of *mstnb* caused abnormal production of serum E2 and VTG

IF and qRT-PCR analyses showed that the protein and mRNA level of Cyp19a1a in the *mstnb*^{-/-} XX fish were significantly down-regulated at 210 dah (Fig. 3A, B, E), while it was significantly up-regulated at 360 dah in comparison with the WT XX fish (Fig. 3C, D, F). Consistently, EIA assay showed that the serum E2 and VTG of *mstnb*^{-/-} XX fish were significantly decreased compared with those of the WT XX fish at 210 dah (Fig. 3G, I). At 360 dah, the serum E2 was significantly increased in *mstnb*^{-/-} XX tilapia. The serum VTG level was comparable to that of WT XX fish at 360 dah (Fig. 3H, J).

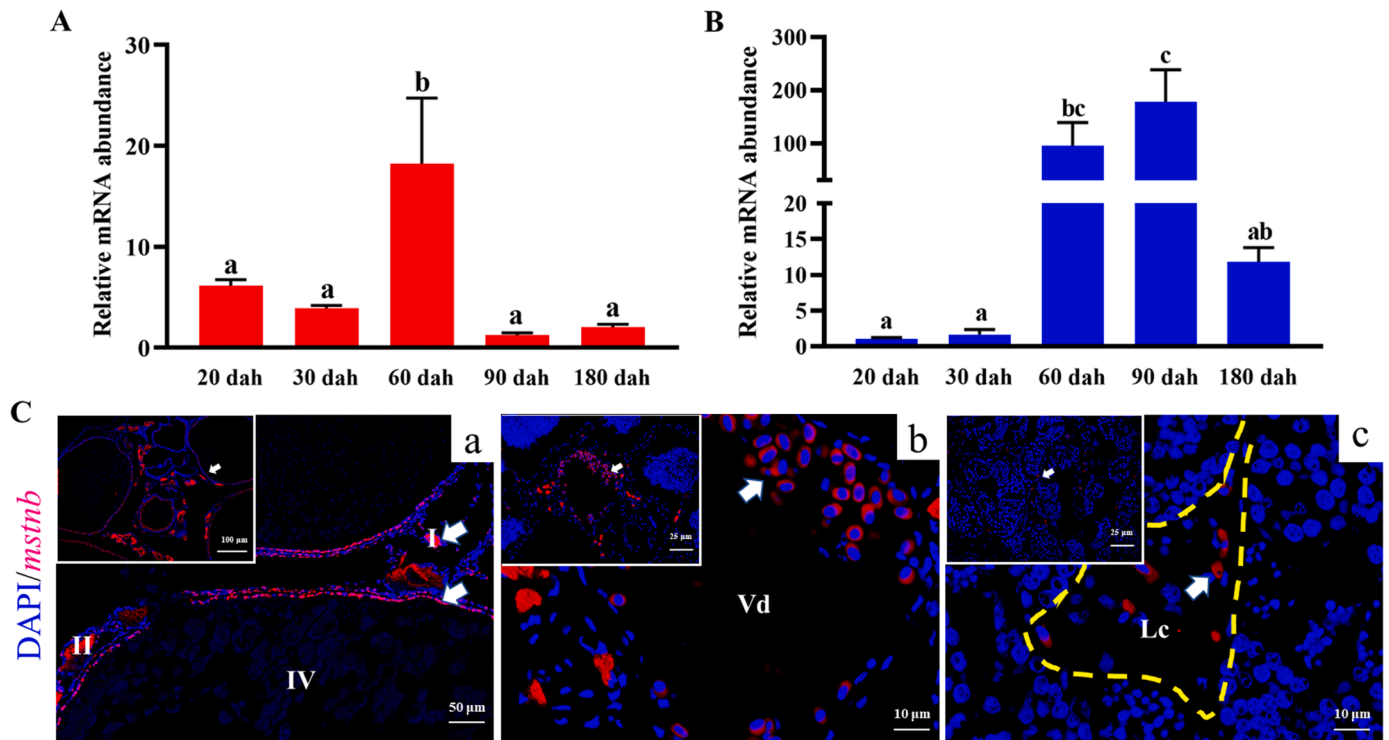


Fig. 1. Immunofluorescence (IF) localization of *mstnb* in tilapia gonad. (A-B) Expression of *mstnb* mRNA in the tilapia ovaries (A) and testes (B) at different developmental stages, as determined by qRT-PCR. Data are expressed as the mean $\pm$ SD of three different gonadal pools at each developmental stage. Different letters above the error bar indicate statistical differences at $p < 0.05$ as determined by one-way ANOVA followed by Tukey test. (C) *mstnb* was mainly expressed in the phase I-II oocytes, follicular cells of late-phase oocytes in the ovary (a), and the vas deferens endothelial cells (b) and Leydig cell (c) of testis. Blue and red indicated the DAPI and *mstnb* positive signaling, respectively. The area surrounded by the yellow dotted line indicated Leydig cells. White arrow indicates the position of positive signaling. I to IV, phase I to IV oocytes; Vd, vas deferens; Lc, Leydig cell; dah, days after hatching.

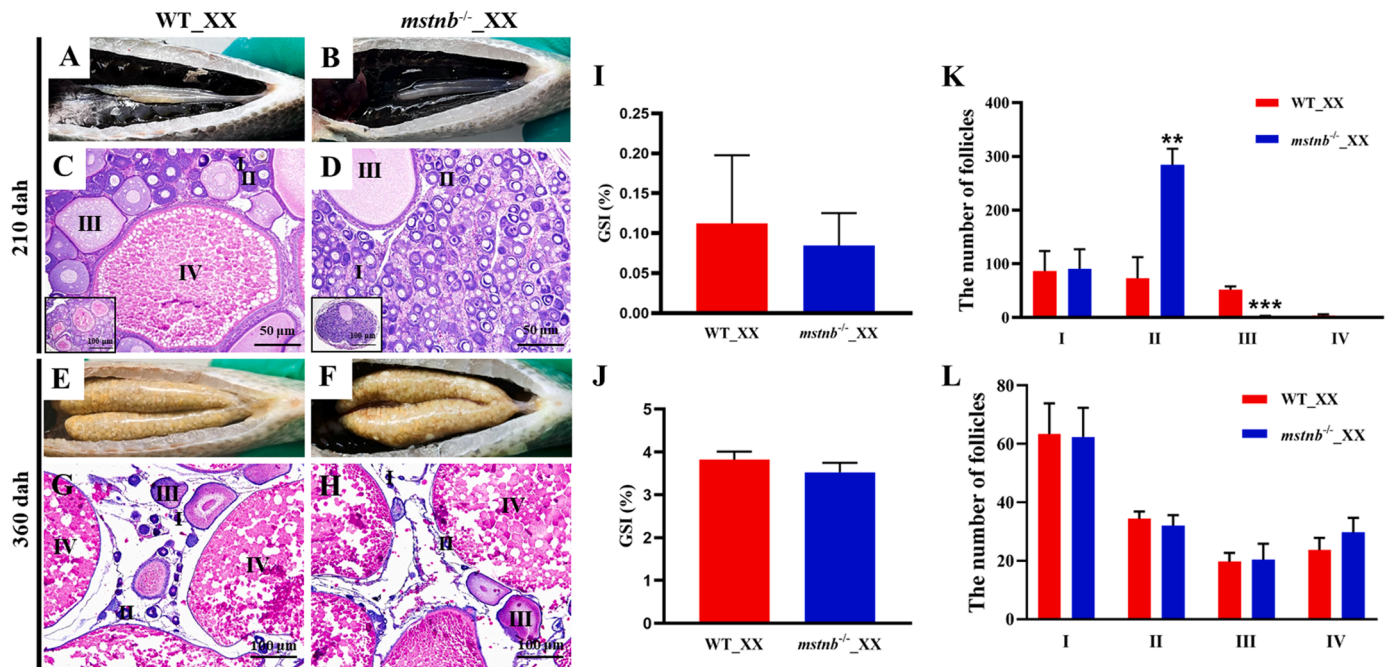


Fig. 2. Effects of homozygous mutation of *mstnb* gene on ovarian development in Nile tilapia at 210 and 360 dah. (A-H) Morphological and histological observation. (I-J) Gonadosomatic index (GSI) (n = 4). (K-L) Statistical analysis of follicles counting (n = 4). Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001. Results were presented as the mean ± SD. I to IV, phase I to phase IV follicles; dah, days after hatching.

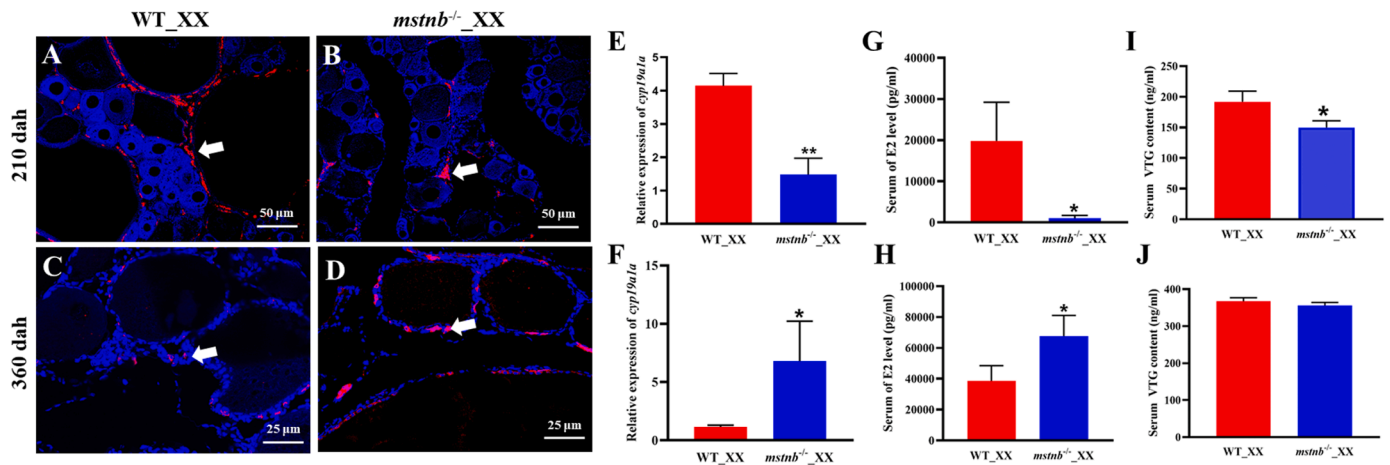


Fig. 3. Homozygous mutation of *mstnb* gene caused abnormal syntheses of E2 and VTG at 210 and 360 dah. (A-D) Immunofluorescence analysis of Cyp19a1a expression in WT and *mstnb*^{-/-} XX fish. Blue and red indicated the DAPI and Cyp19a1a positive signaling, respectively. White arrow indicates the position of positive signaling. (E-F) The expression of *cyp19a1a* in ovaries was detected by qRT-PCR (n = 4). (G-J) Serum E2 and VTG were detected by EIA assay (n = 4). Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, *P < 0.05; **P < 0.01. Results were presented as the mean ± SD. E2, 17β-estradiol; VTG, vitellogenin; dah, days after hatching.

3.4. *Mstnb* mutation impaired testicular development in tilapia

In this study, we conducted experiments to clarify the effects of *mstnb* gene mutation on the development of testes. Morphological analysis showed that the testes of *mstnb*^{-/-} XY fish were thinner and shorter than that of WT XY fish at 210 and 360 dah (Fig. 4A-B, E-F). GSI of *mstnb*^{-/-} XY fish was lower than that of WT XY fish at 210 and 360 dah (Fig. 4I-J). Further histological analysis revealed more spermatogonia in the *mstnb*^{-/-} testes than that in WT testes (Fig. 4C-D, G-H, K-L). Taken together, these results suggested that deficiency of Mstn severely impaired the testicular development in *mstnb*^{-/-} males.

3.5. *Mstnb* mutation caused abnormal synthesis of 11-KT at 210 and 360 dah

Spermatogenesis is a highly organized and coordinated process during which a small number of diploid spermatogonial stem cells, the founder cells of the germ line, develop through mitosis, meiosis and differentiation into the haploid spermatozoa (Chocu et al., 2012; Nishimura and L'Hernault, 2017; Tokalov et al., 2006). Androgens are one of the main hormones involved in spermatogenesis, secondary sexual characteristics and male behaviors in vertebrates (Schulz and Miura, 2002; Smith and Walker, 2014; Walker, 2010). To further understand the reason for the development of the small testes and delayed

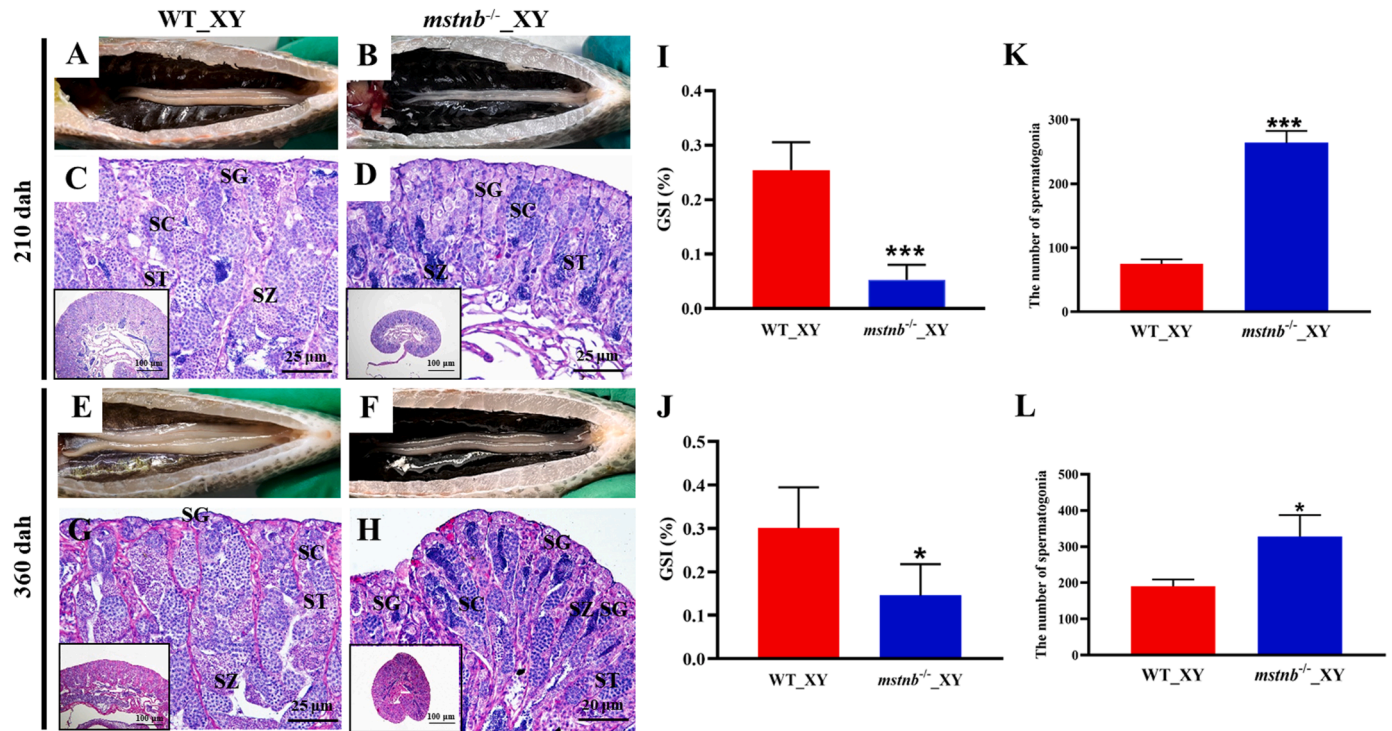


Fig. 4. Effects of homozygous mutation of *mstnb* gene on testicular development in Nile tilapia at 210 and 360 dah. (A-H) Morphological and histological observation. (I-J) Gonadosomatic index (GSI) (n = 4). (K-L) Statistics of the number of spermatogonia (n = 4). Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001. Results were presented as the mean ± SD. SG, spermatogonia; SC, spermatocyte; ST, spermatid; SZ, spermatozoa; dah, days after hatching.

spermatogenesis in *mstnb*^{-/-} XY tilapia, expression of steroidogenic enzyme gene and serum 11-KT level were measured. IF and qRT-PCR analyses showed that the protein and mRNA level of Cyp11b2 in the *mstnb*^{-/-} XY fish were significantly decreased at 210 dah, while the level of Cyp11b2 protein and mRNA were significantly increased at 360 dah in comparison with the WT XY fish (Fig. 5A-F). Consistently, EIA analyses showed that the serum 11-KT of *mstnb*^{-/-} XY fish was significantly

decreased compared with that of the WT XY fish at 210 dah (Fig. 5G). On the contrary, the serum 11-KT content was significantly augmented in *mstnb*^{-/-} XY tilapia at 360 dah (Fig. 5H).

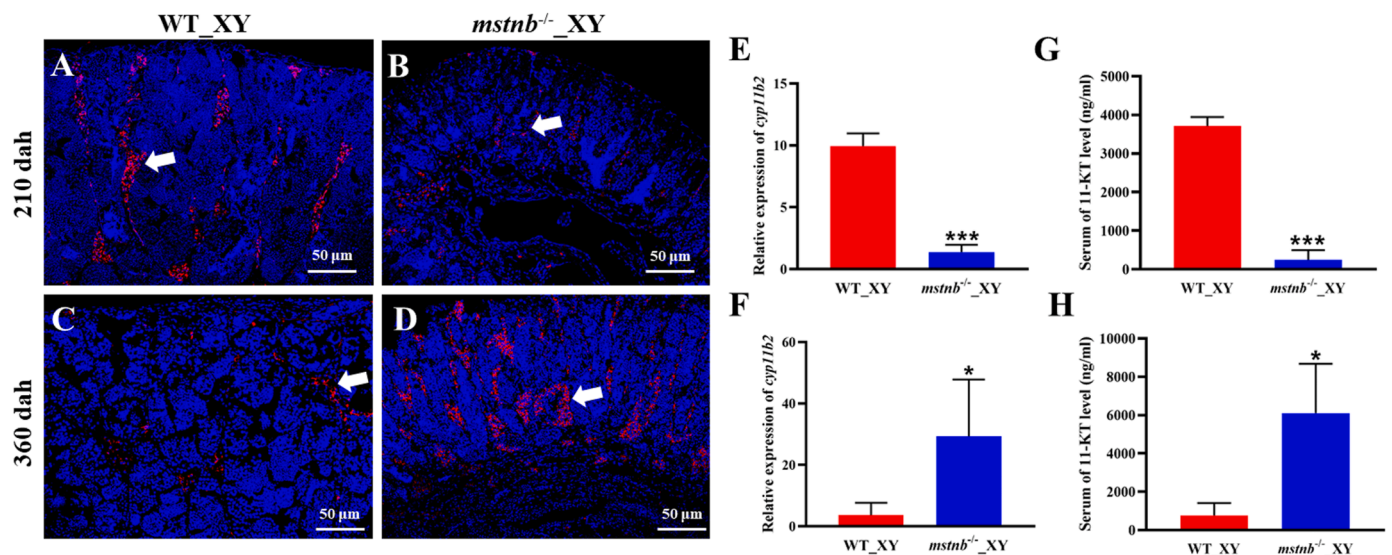


Fig. 5. Homozygous mutation of *mstnb* gene caused abnormal synthesis of 11-KT at 210 and 360 dah. (A-D) Immunofluorescence analysis of Cyp11b2 expression in WT and *mstnb*^{-/-} XY fish. Blue and red indicated the DAPI and Cyp11b2 positive signaling, respectively. White arrow indicates the position of positive signaling. (E-F) The expression of *cyp11b2* in testis was detected by qRT-PCR (n = 4). (G-H) Serum 11-KT level was detected by EIA assay (n = 4). Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001. Results were presented as the mean ± SD. 11-KT, 11-ketotestosterone; dah, days after hatching.

3.6. Effects of *mstnb* gene mutation on fertility in both female and male tilapia

In laboratory condition, adult female tilapia usually spawn every 14 days. In view of the abnormal development of gonads and the disordered sex hormone syntheses in *Mstnb* knockout tilapia, we examined the spawning frequency and offspring survival rate on *mstnb*^{-/-} XX tilapia. Meanwhile, urogenital papilla histological observation for *mstnb*^{-/-} XY tilapia were also conducted to investigate the effects of *mstnb* gene mutation on fertility at 360 dah. The frequency of spawning was detected for four consecutive cycles during 60 days. Similar as the WT XX fish, the *mstnb*^{-/-} XX fish (n = 4) displayed the normal courtship behavior, nesting behaviors, mouth brooding and 14-day spawning cycle (Fig. 6A). However, only one *mstnb*^{-/-} XX female mutant successfully produced a few viable offspring confirmed their poor egg quality (data not shown). And a higher mortality of the offspring obtained by artificial fertilizing the eggs from *mstnb*^{-/-} XX (n = 3) fish and sperm from WT XY fish were detected at 3 days post fertilization. Therefore, we conclude that the *mstnb*^{-/-} female mutants are subfertile, but not sterile (Fig. 6B). In male fish, the semen of WT XY fish (n = 3) can be easily collected by manual extrusion, while the semen of *mstnb*^{-/-} XY fish (n = 3) were unable to be squeezed out. Furthermore, histological observation of urogenital papilla of *mstnb*^{-/-} XY fish were conducted to find out the reason for dyspermastism. The urogenital papilla of *mstnb*^{-/-} XY fish was smaller than that of WT XY fish (Fig. 7A-B). Histological analysis revealed that the smooth muscle thickness in position 1 and position 2 of *mstnb*^{-/-} XY fish urogenital papilla was thinner than that of WT XY fish urogenital papilla (Fig. 7C-D).

It is well accepted that genetic redundancy can confer robustness and evolutionary flexibility to organisms, and a copy of paralogous gene usually compensate for the loss or mutation of another copy. Therefore, qRT-PCR were carried out to further clarify the effects of *mstnb* gene mutation on the expression of its paralogous gene *mstna* at different developmental stages. In females, the results showed that there was no difference in *mstna* gene expression of WT and *mstnb*^{-/-} ovaries at 210 dah, but *mstna* was up-regulated in *mstnb*^{-/-} ovaries compared to WT ovaries at 360 dah (Fig. 8A). In males, the relative expression levels of

mstna in *mstnb*^{-/-} testes were comparable to those of WT testes at both 210 and 360 dah (Fig. 8B). Therefore, the compensation of *mstna* gene expression due to *mstnb* gene deficiency was only detected on adult female fish. Consistently, FISH showed that *mstna* was dominantly expressed in the cytoplasm of phase I to III oocytes in WT ovary (Fig. 8C), while the *mstna* mRNA was not detected in the WT testis (Fig. 8D-E).

3.7. Effects of *mstnb* gene mutation on testicular cell apoptosis

In order to further evaluate the cellular apoptosis in the impaired testis of *mstnb*^{-/-} tilapia, the apoptotic fluorescence signaling of testicular cells were examined in WT and *mstnb*^{-/-} XY fish by TUNEL staining. TUNEL assay showed that there were strong apoptotic signaling in *mstnb*^{-/-} testes at 210 and 360 dah. Significant increase of positive apoptotic signals were observed in both spermatogenic cell and epithelial cells of vas deferens at 210 dah, while the positive signals were concentrated on epithelial cells in *mstnb*^{-/-} testes than that of WT fish at 360 dah (Fig. 9A-F).

4. Discussion

Mstn is a member of the transforming growth factor-beta (TGF- β) superfamily and is primarily known for its role in regulating skeletal muscle development and growth (McPherron et al., 1997). However, emerging research has shed light on its involvement in reproduction as well (Baig et al., 2022; Wang et al., 2022). In the present study, we found that *mstnb* was strongly expressed in the 60 and 90 dah gonads of tilapia. Furthermore, *mstnb* was localized in the phase I-II oocytes and follicular cells of late-phase oocytes of the ovary, and the vas deferens endothelial cells and Leydig cell of testis. Deficiency of *Mstnb* led to the production of poor-quality eggs in females and spermiation obstacle in males. Our data confirmed that *Mstnb* may be a critical factor by regulating steroidogenesis and fertility in tilapia.

Previous studies had showed that MSTN was notably expressed in gonads indicating its potential roles in the reproductive system. In human, GDF8 was expressed in follicular fluid and uterus (Fang et al., 2020). MSTN had also been linked to the regulation of ovarian steroidogenesis, which is important for reproductive hormone production and menstrual cycle regulation in human (Wang et al., 2022). Deficiency of MSTN has been shown to influence ovarian follicle development and oocyte maturation (Chang et al., 2016a; Cheewasopit et al., 2018; Chen et al., 2012; Fang et al., 2015). Supplementation with GDF8 during porcine *in vitro* oocyte maturation enhanced both meiotic and cytoplasmic maturation, while inhibition of GDF8-induced Smad2/3 signaling reduces matured oocyte quality (Yoon et al., 2019). In chicken, *Mstn* was reported to be expressed ubiquitously in many organs of embryos, most notably the testis and ovary (Kubota et al., 2007). In teleosts, *mstn* genes were also expressed in ovary and testis in several species, such as zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and Mozambique tilapia (*Oreochromis mossambicus*) (Helterline et al., 2007; Ostbye et al., 2001; Roberts and Goetz, 2003; Rodgers et al., 2001). However, the specific mechanisms and functions of *Mstn* in fish gonadal development and fertility are largely unknown. In the present study, we found that tilapia *mstnb* was strongly expressed in the 60 and 90 dah the gonads of tilapia, which indicated that *Mstnb* might be involved in the regulation of development of both ovaries and testis. Furthermore, *mstnb* was localized in the cytoplasm of phase I-II oocytes and follicular cells of late-phase oocytes in the ovary, and the Leydig cell of the testis, which confirmed that *Mstnb* might be involved in the regulation of steroidogenesis and fertility in tilapia. Consistently, homozygous mutation of tilapia *mstnb* resulted in retardation of oogenesis, delay of oocyte maturation and poor quality of eggs in females. Similarly, *mstnb* gene mutation in male led to the blockage of spermatogenesis and no sperm was able to be squeezed out. These results indicated that deficiency of *mstnb* gene

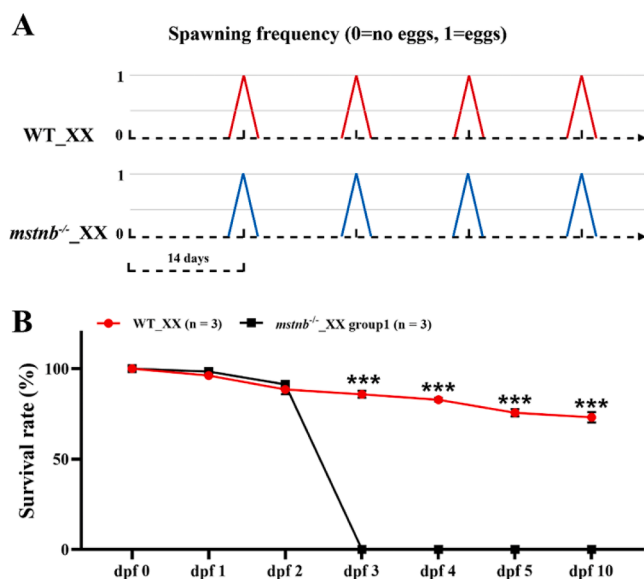


Fig. 6. Fertility tests of WT and *mstnb*^{-/-} XX fish at 360 dah. (A) Spawning frequency of WT (n = 4) and *mstnb*^{-/-} XX (n = 4) fish during four 14-day spawning cycles. (B) Offspring survival rate of WT (n = 3) and *mstnb*^{-/-} (n = 3) XX fish. Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001. Results were presented as the mean $\pm$ SD. dah, days after hatching; dpf, days post fertilization.

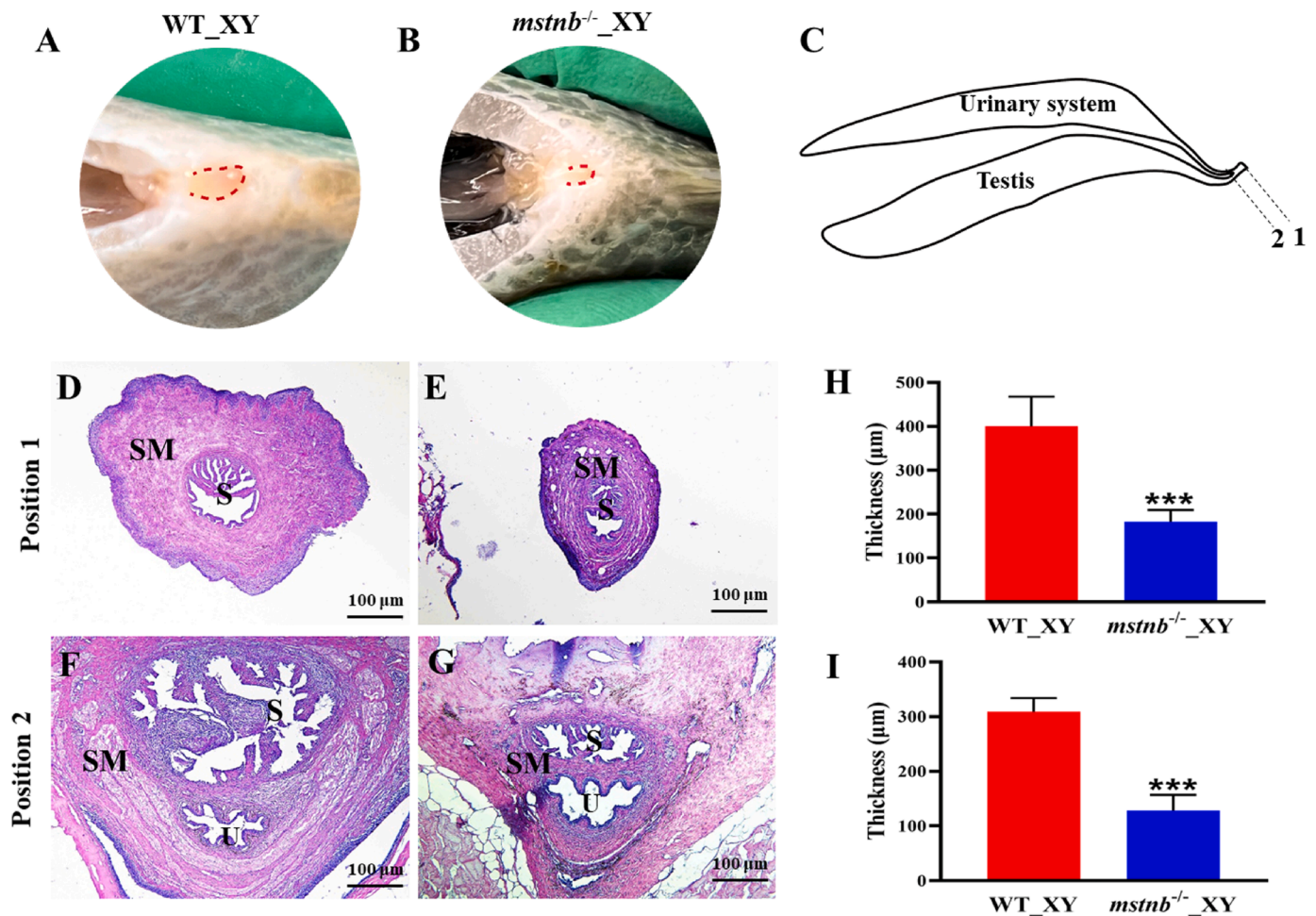


Fig. 7. Urogenital papilla histological observation of WT and *mstnb*^{-/-} XY fish at 360 dah. (A-B) Morphological observation of the urogenital papilla (n = 4). (C) Scheme of the urogenital papilla in Nile tilapia. (D-G) Histological analysis of WT and *mstnb*^{-/-} XY urogenital papilla in different position. (H-I) The thickness of smooth muscle in WT and *mstnb*^{-/-} XY urogenital papilla. Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001. Results were presented as the mean ± SD. S, sperm duct; U, urinary duct; SM, smooth muscle. dah, days after hatching.

caused subfertility in female tilapia and sterility in male tilapia. Therefore, we proved, for the first time, *mstn* gene plays an essential role in the maintenance of fish fertility. It was well known that fish reproduction is critically controlled by the hypothalamus-pituitary-gonad (HPG) axis (Zohar et al., 2010). Undoubtedly, the expression of *mstnb* in the gonads might be required for the gonadal development and functions. Our previous study showed that tilapia *mstnb* was also expressed in brain and pituitary with a relatively lower level (Wu et al., 2023). Therefore, the phenotypes of female subfertility and male sterility in the *mstnb*^{-/-} tilapia might be resulted from the deficiency of Mstn in other reproductive organs in HPG axis, which needs to be further investigated.

Due to the accumulation of yolk protein, the size of oocyte increased several times during oogenesis in teleosts: this process is known as vitellogenesis. It is well accepted that circulating E2 acts on the hepatocytes (liver cells) and regulates the expression of VTG genes, which are transported via the bloodstream to the developing oocytes (eggs) (Gupta et al., 2021). In human granulosa-lutein cells, GDF8 (MSTN) treatment could upregulate the expression of aromatase and the level of E2 suggesting that GDF8 can stimulate estradiol production (Chang et al., 2016a). Consistently, we found female tilapia lacking Mstnb showed significant decrease of serum VTG and E2 levels, which led to the delay of ovarian development and blockage of transition of primary oocytes (phase II) to secondary oocytes (phase III) due to the arrest of vitellogenesis. Furthermore, the dramatic increase of mortality of progenies

produced by *mstnb*^{-/-} female fish suggested that *mstnb* deficiency resulted in poor quality of eggs. Therefore, expression of Mstn in ovaries might play essential roles in fish ovarian development and fertility by promoting VTG and E2 production. The importance of E2-estrogen receptor signaling pathway in fish vitellogenesis had been reported in previous study (Nelson and Habibi, 2013). Yan et al. found that the estrogen receptor 2a (*esr2a*) mutated female tilapias showed a delayed ovarian development at 90 and 180 dah, however, ovarian development caught up later by 360 dah (Yan et al., 2019). Similarly, the delay of ovarian development in *mstnb*^{-/-} tilapia also recovered at 360 dah because of the fact that the serum E2 level have recovered or even exceeded that of the WT XX fish. Meanwhile, the up-regulation of *mstna*, which was expressed in the cytoplasm of phase I to III oocytes of WT ovaries, in *mstnb*^{-/-} female fish might partially contribute to the recovery of ovarian development via germ-somatic cell interaction around 360 dah. These finding suggests that up-regulation of *mstna* might compensate for the deficiency of *mstnb* in follicular cells, which further promote E2 production and ovarian development, ovulation and spawning regularly. However, Mstna compensation only partially restored the fertility due to the poor quality of eggs produced by *mstnb*^{-/-} female fish. Therefore, further investigations are needed to explore the molecular mechanisms on Mstn-activated signaling pathways in fish fertility in future.

Spermatogenesis is a continuous and dynamic developmental process, in which diploid spermatogonial stem cell (SSC) proliferates and differentiates into spermatocytes, and finally form a mature

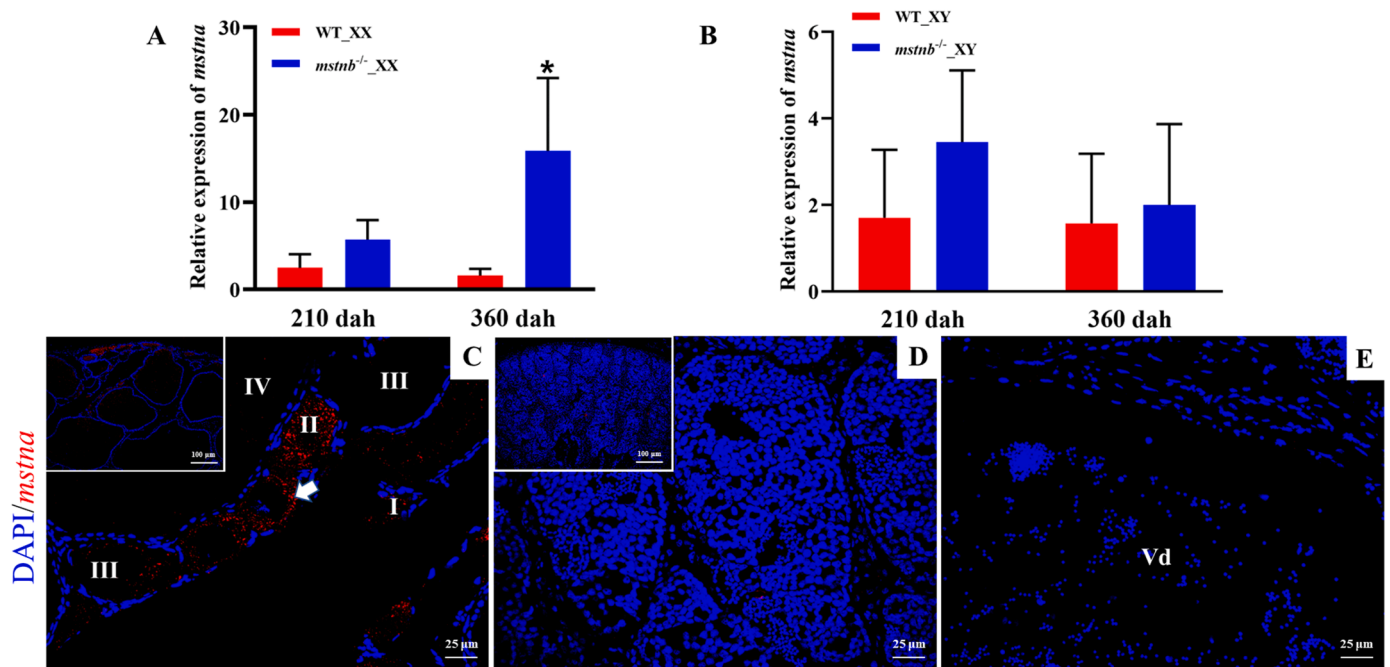


Fig. 8. Relative expression of *mstna* in *mstnb* knockout ovaries and testes at 210 and 360 dah. (A) The expression of *mstna* in ovaries were detected by qRT-PCR ($n = 4$). (B) Relative expression of *mstna* in testes were detected by qRT-PCR ($n = 4$). Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, $*P < 0.05$. Results were presented as the mean $\pm$ SD. (C) *Mstna* was expressed in the cytoplasm of phase I-III oocytes in the ovary. (D-E) There was no *mstna* positive signals in the testis. Blue and red indicated the DAPI and *mstna* positive signaling, respectively. White arrow indicated the position of positive signaling. I to IV, phase I to IV follicles; Vd, vas deferens. dah, days after hatching.

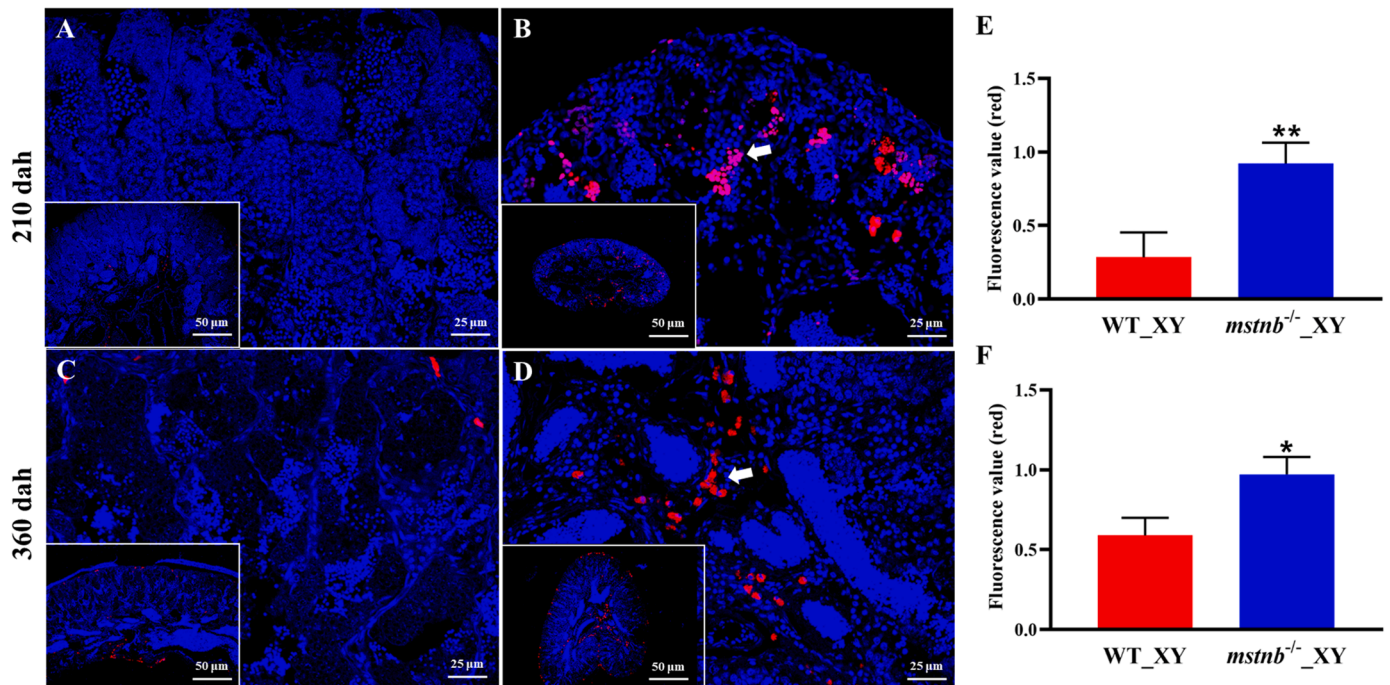


Fig. 9. Homozygous mutation of *mstnb* caused testicular cell apoptosis at 210 and 360 dah. (A-B) The apoptotic signaling of WT (A) and *mstnb*^{-/-} (B) XY fish were detected by TUNEL staining at 210 dah ($n = 3$). The apoptotic signaling of WT (C) and *mstnb*^{-/-} (D) XY fish were detected by TUNEL staining at 360 dah ($n = 3$). (E-F) TUNEL-positive fluorescence value at 210 (E) and 360 (F) dah were quantified by image J. Blue and red indicate the DAPI and apoptosis positive signaling, respectively. White arrow indicates the position of positive signaling. dah, days after hatching.

spermatozoon (Xie et al., 2020). Androgens, produced by Leydig cells in the testis, play a crucial role in supporting and regulating spermatogenesis (Ge et al., 2021). In fish, 11-KT has been proved to be the primary androgen and acts as a key regulator of various stages of sperm cell

development, including the proliferation and differentiation of germ cells, meiosis, and sperm maturation (Alam et al., 2006; Miura et al., 1991; Reinboth, 1975). Recent studies revealed that deficiency of Cyp11b2, encoding one of the key steroidogenic enzymes for androgen

production, exhibited a sharp decrease in serum 11-KT, which further resulted in the delay of spermatogenesis, development of smaller testes and efferent duct in tilapia (Zheng et al., 2020). In the present study, we found that mutation of *mstnb* led to dramatic decline of serum 11-KT level and expression of *cyp11b2* gene in XY tilapia at 210 dah, which might account for the delay of spermatogenesis and development of smaller testis. Interestingly, serum 11-KT level and expression of *cyp11b2* were significantly upregulated at 360 dah. These data suggested that Mstnb played a crucial role in the maintenance of spermatogenesis, possibly by regulating the biosynthesis of 11-KT. However, failure of semen extrusion and a significant increase in apoptotic testicular epithelia cells were observed in *mstnb*^{-/-} XY mutants suggesting a significant impairment in the reproductive function, particularly in the process of sperm release.

Our findings provided compelling evidences for the crucial role of Mstn in teleost gonadal development and fertility. Particularly, we observed that Mstnb played a significant role in promoting oogenesis and the development of high-quality eggs in female fish, which might be achieved through regulating E2 biosynthesis in the follicular cells, VTG release and incorporation into the oocytes. In male fish, Mstnb was essential for spermatogenesis and testicular differentiation by controlling the production of 11-KT, as well as the maintenance of testicular functions. Overall, our study sheds new light on the regulatory roles of Mstn in fish fertility, which contribute to our understanding of the networks of reproductive regulation in teleosts. In addition, we speculated that sub-sterility and infertility of *mstn*^{-/-} mutants might partially promote the significant increase of body growth due to the trade-off between body growth and fecundity. However, the mechanisms of the trade-off need to be investigated by measuring the changes of both serum glucose and lipids. Our data have the potential to promote advancements in aquaculture and fisheries management by enabling the production of *mstn*^{-/-} sterile strains.

Supplemental figure legends

Supplemental Fig. 1 The identification of genetic sex of both WT and *mstnb*^{-/-} Nile tilapia.

A pair of sex-specific primers (SPF/SPR) was performed to PCR amplification. XX (female): one band; XY (male): two bands.

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CRediT authorship contribution statement

Zhao Chenhua: Data curation, Resources. **Li Lu:** Data curation. **Cai Jing:** Methodology, Validation. **Wang Deshou:** Funding acquisition, Project administration. **Zhou linyan:** Conceptualization, Funding acquisition, Methodology, Project administration. **Wu You:** Formal analysis, Investigation, Writing – original draft. **Yang Lanying:** Formal analysis, Investigation. **Du Yiyun:** Formal analysis, Visualization. **Su Yun:** Data curation, Resources.

Declaration of Competing Interest

The authors declare that there is no financial or other potential conflict of interests.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.101926.

References

- Alam, M.A., Bhandari, R.K., Kobayashi, Y., Nakamura, S., Soyano, K., Nakamura, M., 2006. Changes in androgen-producing cell size and circulating 11-ketotestosterone level during female-male sex change of honeycomb grouper *Epinephelus merra*. *Mol. Reprod. Dev.* 73, 206–214.
- Baig, M.H., Ahmad, K., Moon, J.S., Park, S.Y., Lim, J.H., Chun, H.J., Qadri, A.F., Hwang, Y.C., Jan, A.T., Ahmad, S.S., et al., 2022. Myostatin and its regulation: a comprehensive review of myostatin inhibiting strategies. *Front. Physiol.* 13.
- Bartley, D.M., Rana, K., Immink, A.J., 2000. The use of inter-specific hybrids in aquaculture and fisheries. *Rev. Fish. Biol. Fish.* 10, 325–337.
- Brawand, D., Wagner, C.E., Li, Y.L., Malinsky, M., Keller, I., Fan, S.H., Simakov, O., Ng, A. Y., Lim, Z.W., Bezaul, E., et al., 2014. The genomic substrate for adaptive radiation in African cichlid fish. *Nature* 513, 375–381.
- Chang, H.M., Fang, L., Cheng, J.C., Klausen, C., Sun, Y.P., Leung, P.C., 2015b. Growth differentiation factor 8 down-regulates pentraxin 3 in human granulosa cells. *Mol. Cell Endocrinol.* 404, 82–90.
- Chang, H.M., Fang, L., Cheng, J.C., Taylor, E.L., Sun, Y.P., Leung, P.C., 2016a. Effects of growth differentiation factor 8 on steroidogenesis in human granulosa-lutein cells. *Fertil. Steril.* 105, 520–528.
- Cheewasopit, W., Laird, M., Glister, C., Knight, P.G., 2018. Myostatin is expressed in bovine ovarian follicles and modulates granulosa and thecal steroidogenesis. *Reproduction* 156, 375–386.
- Chen, M.J., Han, D.S., Yang, J.H., Yang, Y.S., Ho, H.N., Yang, W.S., 2012. Myostatin and its association with abdominal obesity, androgen and follistatin levels in women with polycystic ovary syndrome. *Hum. Reprod.* 27, 2476–2483.
- Chocui, S., Calvel, P., Rolland, A.D., Pineau, C., 2012. Spermatogenesis in mammals: proteomic insights. *Syst. Biol. Reprod. Med.* 58, 179–190.
- Coward, K., Bromage, N.R., 1998. Histological classification of oocyte growth and the dynamics of ovarian recrudescence in Tilapia zillii. *J. Fish. Biol.* 53, 285–302.
- Fang, L., Chang, H.-M., Cheng, J.C., Yu, Y., Leung, P.C.K., Sun, Y.P., 2015. Growth differentiation factor-8 decreases StAR expression through ALK5-mediated Smad3 and ERK1/2 signaling pathways in luteinized human granulosa cells. *Endocrinology* 156, 4684–4694.
- Fang, L.L., Wang, S.J., Li, Y.R., Yu, Y.P., Li, Y.X., Yan, Y., Cheng, J.C., Sun, Y.P., 2020. High GDF-8 in follicular fluid is associated with a low pregnancy rate in IVF patients with PCOS. *Reproduction* 160, 11–19.
- Ge, R.S., Li, X.H., Wang, Y.Y., 2021. Leydig cell and spermatogenesis. *Adv. Exp. Med Biol.* 1288, 111–129.
- Gupta, K., Kumar, M., Rani, S., Mohanta, B., 2021. Vitellogenesis and their endocrine control in fishes. In: Sundaray, J.K., Rather, M.A., Kumar, S., Agarwal, D. (Eds.), *In recent updates in molecular endocrinology and reproductive physiology of fish: an imperative step in aquaculture*. Springer Singapore, Singapore, pp. 23–34.
- Hallerman, E.M., Kapuscinski, A.R., 1995. Incorporating risk assessment and risk management into public policies on genetically modified finfish and shellfish. *Aquaculture* 137, 9–17.
- Helterline, D.L.I., Garikipati, D., Stenkamp, D.L., Rodgers, B.D., 2007. Embryonic and tissue-specific regulation of myostatin-1 and -2 gene expression in zebrafish. *Gen. Comp. Endocr.* 151, 90–97.
- Hull, K.L., Harvey, S., 2014. Growth hormone and reproduction: a review of endocrine and autocrine/paracrine interactions. *Int. J. Endocrinol.* 2014.
- Kubota, K., Sato, F., Aramaki, S., Soh, T., Yamauchi, N., Hattori, M.A., 2007. Ubiquitous expression of myostatin in chicken embryonic tissues: its high expression in testis and ovary. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 148, 550–555.
- Li, H., Wang, G., Hao, Z., Zhang, G., Qing, Y., Liu, S., Qing, L., Pan, W., Chen, L., Liu, G., et al., 2016. Generation of biallelic knock-out sheep via gene-editing and somatic cell nuclear transfer. *Sci. Rep.* 6, 33675.
- Li, M., Wu, F., Gu, Y., Wang, T., Wang, H., Yang, S., Sun, Y., Zhou, L., Huang, X., Jiao, B., et al., 2012. Insulin-like growth factor 3 regulates expression of genes encoding steroidogenic enzymes and key transcription factors in the Nile tilapia gonad. *Biol. Reprod.* 86, 161–110.
- Li, M.H., Sun, Y.L., Zhao, J.E., Shi, H.J., Zeng, S., Ye, K., Jiang, D.N., Zhou, L.Y., Sun, L. N., Tao, W.J., et al., 2015. A tandem duplicate of anti-müllerian hormone with a missense SNP on the Y chromosome is essential for male sex determination in Nile tilapia, *Oreochromis niloticus*. *Plos Genet* 11.
- Li, M.H., Liu, X.Y., Dai, S.F., Xiao, H.S., Qi, S.S., Li, Y.B., Zheng, Q.Y., Jie, M.M., Cheng, C.H.K., Wang, D.S., 2020. Regulation of spermatogenesis and reproductive capacity by Igf3 in tilapia. *Cell Mol. Life Sci.* 77, 4921–4938.
- McPherron, A.C., Lawler, A.M., Lee, S.J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387, 83–90.
- Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *P. Natl. Acad. Sci.* 88, 5774–5778.

- Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. *Gen. Comp. Endocr.* 192, 15–24.
- Neves, P.R., Natali, M.R.M., Ribeiro, R.P., Vargas, L., Maehana, K.R., Marengoni, N.G., 2009. Morphological characteristics of ovarian development of two Nile tilapia (*Oreochromis niloticus*) strains in mixed-culture systems. *Arq. Bras. De. Med. Vet. E Zootec.* 61, 1173–1182.
- Nishimura, H., L'Hernault, S.W., 2017. Spermatogenesis. *Curr. Biol.* 27, R988–R994.
- Ohama, M., Washio, Y., Kishimoto, K., Kinoshita, M., Kato, K., 2020. Growth performance of myostatin knockout red sea bream *Pagrus major* juveniles produced by genome editing with CRISPR/Cas9. *Aquaculture* 529.
- Ostbye, T.K., Galloway, T.F., Nielsen, C., Gabestad, I., Bardal, T., Andersen, O., 2001. The two myostatin genes of Atlantic salmon (*Salmo salar*) are expressed in a variety of tissues. *Eur. J. Biochem* 268, 5249–5257.
- Qian, L.L., Tang, M.X., Yang, J.Z., Wang, Q.Q., Cai, C.B., Jiang, S.W., Li, H.G., Jiang, K., Gao, P.F., Ma, D.Z., et al., 2015b. Targeted mutations in myostatin by zinc-finger nucleases result in double-muscling phenotype in Meishan pigs. *Sci. Rep.* 5.
- Rahman, M.A., Uehara, T., Lawrence, J.M., 2005. Growth and heterosis of hybrids of two closely related species of Pacific sea urchins (*Genus Echinometra*) in Okinawa. *Aquaculture* 245, 121–133.
- Reinboth, R., 1975. In vitro studies on steroid metabolism of testicular tissue in ambisexual teleost fish. *J. Steroid Biochem.* 6, 341–344.
- Roberts, S.B., Goetz, R.W., 2003. Myostatin protein and RNA transcript levels in adult and developing brook trout. *Mol. Cell Endocrinol.* 210, 9–20.
- Rodgers, B.D., Weber, G.M., Sullivan, C.V., Levine, M.A., 2001. Isolation and characterization of myostatin complementary deoxyribonucleic acid clones from two commercially important fish: *Oreochromis mossambicus* and *Morone chrysops*. *Endocrinology* 142, 1412–1418.
- Roff, D.A., 2000. Trade-offs between growth and reproduction: an analysis of the quantitative genetic evidence. *J. Evol. Biol.* 13, 434–445.
- Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. *Fish. Physiol. Biochem.* 26, 43–56.
- Skinner, M.K., Schmidt, M., Savenkova, M.I., Sadler-Riggelman, I., Nilsson, E.E., 2008. Regulation of granulosa and theca cell transcriptomes during ovarian antral follicle development. *Mol. Reprod. Dev.* 75, 1457–1472.
- Smith, L.B., Walker, W.H., 2014. The regulation of spermatogenesis by androgens. *Semin Cell Dev. Biol.* 30, 2–13.
- Sun, Y.L., Jiang, D.N., Zeng, S., Hu, C.J., Ye, K., Yang, C., Yang, S.J., Li, M.H., Wang, D.S., 2014. Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 433, 19–27.
- Tokalov, S.V., Pfennig, F., Gutzeit, H.O., 2006. Spermatogenesis in fish. *Eur. J. Cell Biol.* 85, 31–31.
- UmEKalsoom, Salim, Shahzadi, M., Barlas, A. T., 2009. Growth performance and feed conversion ratio (Fcr) in hybrid fish (*Catla Catla* X *Labeo rohita*) fed on wheat bran, rice broken and blood meal. *Pak. Vet. J.* 29, 55–58.
- Vaughan, D., Ritvos, O., Mitchell, R., Kretz, O., Lalowski, M., Amthor, H., Chambers, D., Matsakas, A., Pasternack, A., Collins-Hooper, H., et al., 2020. Inhibition of Activin/Myostatin signaling induces skeletal muscle hypertrophy but impairs mouse testicular development. *Eur. J. Transl. Myol.* 30, 62–78.
- Walker, W.H., 2010. Non-classical actions of testosterone and spermatogenesis. *Philos. T R. Soc. B* 365, 1557–1569.
- Wang, D.S., Kobayashi, T., Zhou, L.Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K.I., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding protein/steroidogenic factor 1. *Mol. Endocrinol.* 21, 712–725.
- Wang, S.J., Fang, L.L., Cong, L.P., Chung, J.P.W., Li, T.C., Chan, D.Y.L., 2022. Myostatin: a multifunctional role in human female reproduction and fertility - a short review. *Reprod. Biol. Endocrin* 20.
- Wu, Y., Wu, T.F., Yang, L.Y., Su, Y., Zhao, C.H., Li, L., Cai, J., Dai, X.Y., Wang, D.S., Zhou, L.Y., 2023. Generation of fast growth Nile tilapia (*Oreochromis niloticus*) by myostatin gene mutation. *Aquaculture* 562.
- Xie, X., Nobrega, R., Psenicka, M., 2020. Spermatogonial stem cells in fish: characterization, isolation, enrichment, and recent advances of in vitro culture systems. *Biomolecules* 10.
- Yan, L.X., Feng, H.W., Wang, F.L., Lu, B.Y., Liu, X.Y., Sun, L.N., Wang, D.S., 2019. Establishment of three estrogen receptors (*esr1*, *esr2a*, *esr2b*) knockout lines for functional study in Nile tilapia. *J. Steroid Biochem.* 191.
- Yan, R.G., Yang, Q.L., Yang, Q.E., 2020. E4 Transcription factor 1 (E4F1) regulates Sertoli cell proliferation and fertility in mice. *Animals* 10.
- Yoon, J.D., Hwang, S.U., Kim, M., Jeon, Y., Hyun, S.H., 2019. Growth differentiation factor 8 regulates SMAD2/3 signaling and improves oocyte quality during porcine oocyte maturation in vitro. *Biol. Reprod.* 101, 63–75.
- Zhang, X.C., Wang, F., Dong, Z.J., Dong, X.H., Chi, J., Chen, H.G., Zhao, Q.S., Li, K.B., 2020. A new strain of yellow catfish carrying genome edited myostatin alleles exhibits double muscling phenotype with hyperplasia. *Aquaculture* 523.
- Zhao, Y., Yang, L., Su, G., Wei, Z., Liu, X., Song, L., Hai, C., Wu, D., Hao, Z., Wu, Y., et al., 2022. Growth traits and sperm proteomics analyses of myostatin gene-edited Chinese yellow cattle. *Life* 12, 627.
- Zheng, Q.Y., Xiao, H.S., Shi, H.J., Wang, T.R., Sun, L.N., Tao, W.J., Kocher, T.D., Li, M.H., Wang, D.S., 2020. Loss of Cyp11c1 causes delayed spermatogenesis due to the absence of 11-ketotestosterone. *J. Endocrinol.* 244, 487–499.
- Zhou, L.Y., Li, M.H., Wang, D.S., 2021. Role of sex steroids in fish sex determination and differentiation as revealed by gene editing. *Gen. Comp. Endocr.* 313.
- Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocr.* 165, 438–455.