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Loss of Cyp11c1 causes delayed spermatogenesis due to the absence of 11-ketotestosterone

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Abstract

The impacts of androgens and glucocorticoids on spermatogenesis have intrigued scientists for decades. 11 β -hydroxylase, encoded by *cyp11c1*, is the key enzyme involved in the synthesis of 11-ketotestosterone and cortisol, the major androgen and glucocorticoid in fish, respectively. In the present study, a Cyp11c1 antibody was produced. Western blot and immunohistochemistry showed that Cyp11c1 was predominantly expressed in the testicular Leydig cells and head kidney interrenal cells. A mutant line of *cyp11c1* was established by CRISPR/Cas9. Homozygous mutation of *cyp11c1* caused a sharp decrease of serum cortisol and 11-ketotestosterone, and a delay in spermatogenesis which could be rescued by exogenous 11-ketotestosterone or testosterone, but not cortisol treatment. Intriguingly, this spermatogenesis restored spontaneously, indicating compensatory effects of other androgenic steroids. In addition, loss of Cyp11c1 led to undersized testes with a smaller efferent duct and disordered spermatogenic cysts in adult males. However, a small amount of viable sperm was produced. Taken together, our results demonstrate that *cyp11c1* is important for testicular development, especially for the initiation and proper progression of spermatogenesis. 11-ketotestosterone is the most efficient androgen in tilapia.

Key Words

- *cyp11c1*
- 11-ketotestosterone
- delayed spermatogenesis
- undersized testis
- less semen
- CRISPR/Cas9

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Introduction

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 (Schulz *et al.* 2010, Chocu *et al.* 2012, Nishimura & L'Hernault 2017). Androgens are among the main hormones involved in spermatogenesis and male sexual development in vertebrates (Schulz & Miura 2002, Walker 2010, Smith & Walker 2014). The biological functions of androgens are primarily

mediated by the androgen receptor (AR), a ligand-induced nuclear receptor, through binding to androgen response elements (AREs) in the regulatory regions of target genes (Bennett *et al.* 2010, O'Hara & Smith 2015).

Androgens are synthesized from cholesterol by steroid synthases in a complex pathway that is largely conserved among vertebrates (Miller 1988). Despite the conservation in steroid synthesis, preferences for androgen use were found in vertebrates. In mammals, testosterone is thought to be the predominant androgen in testis

(Smith & Walker 2014). Testosterone alone could maintain spermatogenesis *in vitro* (Cunningham & Huckins 1979). Disruption of testosterone production by hypophysectomy, Leydig cell ablation, luteinizing hormone receptor mutation or *cyp17a1* deletion blocked spermatogenesis in mice and rats (Russell & Clermont 1977, Bartlett *et al.* 1986, Zhang *et al.* 2001, Liu *et al.* 2005). In fish, 11-ketotestosterone (11-KT) is generally considered to be a more effective androgen, notwithstanding the high levels of serum testosterone (Nakamura & Nagahama 1989, Borg 1994). The role 11-KT in fish spermatogenesis became clear for the first time in 1991, using an organ culture system *in vitro* (Miura *et al.* 1991), and then repeatedly confirmed in different species (Kobayashi *et al.* 1991, Cavaco *et al.* 1998, Amer *et al.* 2001). 11 β -Hydroxylase, encoded by *cyp11c1*, is the key enzyme involved in 11-KT synthesis. Elevated expression of 11 β -hydroxylase results in high levels of 11-KT during the crucial period of spermatogenesis (Kusakabe *et al.* 2002, Rajakumar & Senthilkumaran 2015). Although the androgenic potency of 11-KT has been determined in various species (Kobayashi *et al.* 1991, Miura *et al.* 1991, Cavaco *et al.* 1998, Amer *et al.* 2001, de Waal *et al.* 2008, Bain *et al.* 2015), the precise role of 11-KT in spermatogenesis remains to be elucidated by a genetic approach.

Cortisol, another steroid synthesized by 11 β -hydroxylase, has been regarded as the main glucocorticoid in teleost fish, linking external environmental stimuli with internal physiological responses during sexual development (Mommensen *et al.* 1999, Todd *et al.* 2016). Although several hypotheses exist, studies on the exact ways in which cortisol may act as a mediator in the masculinization process are still limited (Consten *et al.* 2002, Hayashi *et al.* 2010, Fernandino *et al.* 2012). It is worth mentioning that cortisol was demonstrated to promote spermatogenesis by inducing spermatogonial proliferation through the synthesis of 11-KT in Japanese eel testis explants (Ozaki *et al.* 2006). This still needs to be verified *in vivo*.

Nile tilapia (*Oreochromis niloticus*) is farmed worldwide. It has an XX/XY sex-determination system. The availability of genetic unisexual fish (Sun *et al.* 2014), 14-day spawning cycle, together with high-quality genome sequences (Conte *et al.* 2017) and well-established reverse genetics approaches (Li *et al.* 2014), make tilapia an ideal model to investigate the role of steroids in spermatogenesis. In our previous study we cloned *cyp11c1* and analyzed its expression in Nile tilapia (Zhang *et al.* 2010). To further decipher the role of 11-KT and cortisol in spermatogenesis, we used CRISPR/Cas9 to generate a

line mutant for *cyp11c1* and conducted a comprehensive assessment of *cyp11c1* mutant males, including gonadal phenotypes, serum hormone levels, sperm quality and fecundity. Our research provides a better understanding of the role of 11-KT and cortisol in fish spermatogenesis.

Materials and methods

Animals

Nile tilapia (*Oreochromis niloticus*) were kept in aerated recirculating freshwater tanks at 26°C under a natural photoperiod. All XX progenies were obtained by crossing XX pseudomales (producing sperm after hormonal sex reversal) with XX females. All XY progenies were obtained by crossing YY supermales with XX females. Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

Production and characterization of Cyp11c1 polyclonal antibody

The recombinant construct of *cyp11c1* was prepared by cloning *cyp11c1* coding sequence into the *Eco*RI and *Sal* I restriction site of the pCold I prokaryotic expression vector with His-tag at the N-terminus. This recombinant plasmid was then transformed into *Escherichia coli*, followed by IPTG induction (1 mmol/L). The His-Cyp11c1 recombinant protein (25–30 μ g), purified with a Ni-NTA super flow cartridge (Qiagen), was subsequently used as antigen to immunize rabbits three times at 15-day intervals. Ten days after the last immunization, rabbit blood was collected, and Cyp11c1-specific antibody was purified by affinity chromatography on Sepharose 4B Fast Flow resin (Sigma) with a stock concentration of 2 mg/mL. The antibody specificity was subsequently evaluated by Western blot at a dilution of 1:500. The negative control (NC) was incubated with pre-adsorbed antisera with purified Cyp11c1 protein. Primers used in this experiment are listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Cellular localization of Cyp11c1

Immunohistochemistry (IHC) was performed to determine cellular localization as described previously

(Zhang *et al.* 2010). After anesthesia (250 mg/L, MS-222, Sigma), testes from 5, 30, 90 and 180 dah (days after hatching) fish and head kidneys from 1, 30, 90 and 180 dah fish were collected and fixed immediately in Bouin's fixative solution for 24 h at room temperature. Samples were then dehydrated and embedded in paraffin. Tissue blocks were serially sectioned at 5 μ m. Antibody against Cyp11c1 was diluted at 1:500 for use.

Disruption of *cyp11c1* by CRISPR/Cas9

The sequence of *cyp11c1* (ENSONIG00000006462) was obtained from Ensembl (<http://www.ensembl.org/index.html>). The target site in the coding region of the first exon of *cyp11c1* was selected with an online software package (<http://zifit.partners.org/ZiFiT/>), and the gRNA (guide RNA) and cas9 mRNA were synthesized as previously reported (Li *et al.* 2014). Embryos at the one-cell stage were microinjected with gRNA and Cas9 mRNA with a final concentration of 250 ng/ μ L and 500 ng/ μ L, respectively. F0 mutants were screened by *Mlu*CI restriction enzyme digestion and Sanger sequencing. F1 progeny were produced by crossing F0 chimeric XY males with WT XX females. Siblings carrying a 7 bp deletion at the same locus were intercrossed to produce F2 progeny. PAGE was used to identify mutants in the F2 generation. The genetic sex of each fish was identified by sex-linked marker as described previously (Sun *et al.* 2014). Primers used for screening are listed in Supplementary Table 1.

qPCR

Pituitaries and testes were collected from *cyp11c1*^{+/+} and *cyp11c1*^{-/-} fish at 360 dah ($n=4$ /genotype). RNA was isolated for each replicate using RNAiso Plus (Takara). 500 ng RNA was used for cDNA synthesis with PrimeScript RT Master Mix Perfect Real Time Kit (Takara). Gene expression was analyzed by qPCR (quantitative real-time PCR) using ABI7500 Detection system. All experiments were performed according to the manufacturer's instructions. Gene expression was normalized to *gapdh* using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2000). Primers used for quantitation are listed in Supplementary Table 1.

Western blot

Total protein, extracted from various tissues of adult WT fish and gonads of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} XY fish at 180 dah, was diluted to a final concentration of 20 mg/mL.

A Western blot (WB) was performed as described previously (Li *et al.* 2012) using anti-Cyp11c1 antibody diluted 1:500. The abundance of α -tubulin was examined as a loading control using rabbit anti- α -tubulin with a dilution ratio 1:1000 (Abcam, AB2619646). Horseradish peroxidase-conjugated goat anti-rabbit antibody (Beyotime, Shanghai, China, A0208) was diluted 1:1000 as secondary antibody. Signal was detected with Western Bright ECL Kit (Advansta, CA, USA, lot 19011532) and visualized on a Fusion FX7 (Vilber Lourmat, East Sussex, France).

Histological analysis

The gonadosomatic index (GSI) of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} fish at 360 dah was calculated as (gonad weight/body weight) $\times$ 100% ($n=4$ /genotype). Testes processed for histology were embedded in paraffin after fixation in Bouin's solution (24 h, RT). Embedded samples were then cut serially into slices of 5 μ m for hematoxylin and eosin (H&E) staining and immunohistochemistry as described previously (Zhang *et al.* 2010). The anti-PH3 (Phospho S10 of histone H3, Abcam, ab80612) and anti-Cyp17a2 (2 mg/mL) polyclonal antibody were used to assess the impacts of *cyp11c1* mutation on cell proliferation and steroidogenic properties, respectively, with a 1:1000 dilution. All sections were imaged using an Olympus BX51 optical microscope (Olympus). The relative areas of efferent ducts and Leydig cells (Cyp17a2-positive) in testes from fish at 360 dah were calculated by Image J Pro 1.51 (mid-section, $n=4$ /genotype).

Semen analysis and fecundity evaluation

Semen from *cyp11c1*^{+/+} and *cyp11c1*^{-/-} XY fish (360 dah) was evaluated comprehensively ($n=5$ /genotype). Sperm morphology and motility, including VSL (straight line velocity), VCL (curvilinear velocity) and BCF (beat cross frequency), were assessed using the Sperm Quality Analyzer (Zoneking Software, Beijing, China). Sperm motility was finally graded as A (rapid motion forward), B (slow motion forward), C (no forward motion) or D (hyperslow or motionless). To further check the sperm-fertilizing ability of *cyp11c1*^{-/-} XY fish, an excess of semen from *cyp11c1*^{+/+} and *cyp11c1*^{-/-} XY fish was used to inseminate eggs from WT XX fish.

Rescue experiments

After randomized grouping, the *cyp11c1*^{-/-} XY fish were fed with a diet sprayed with 95% ethanol containing

11-KT, testosterone, cortisol at a 200 µg/g dosage respectively or 95% ethanol only (ctrl). Treatment were administrated from 30 to 75 dah, fish were then supplied with a normal diet until sampling at 90 dah. Thereafter, treatment was evaluated by H&E staining. All images were captured by Olympus BX51 optical microscope (Olympus). Spermatogenic cells including spermatogonia, spermatocytes, and spermatids from the median cross section of the testes were further quantified manually ($n=5$ /administration), primarily based on cell size, nuclear characteristics (degree of chromosome condensation) and location in testis.

EIA measurement of serum 11-KT, testosterone, cortisol and DHT level

After anesthesia, blood was collected from the caudal vein of *cyp11c1*^{+/+}, *cyp11c1*^{+/-} and *cyp11c1*^{-/-} XY fish at 90 and 180 dah. After overnight storage at 4°C, more than 100 µL of serum was collected by centrifugation (2656 g, 10 min, 4°C) and stored at -80°C until use. Serum 11-KT, testosterone, cortisol (Cayman) and 5α-dihydrotestosterone (DHT, Spbio, Wuhan, China) were analyzed using Enzyme Immunoassay (EIA) Kit according to the manufacturer's instructions. The sensitivity of the kit for 11-KT, testosterone, cortisol and DHT is 1.3, 6.0, 35.0 and 14.7 pg/mL, respectively. 50 µl diluted serum (based on the assay sensitivity of each kit) was used for each well. All samples (serum from single fish as one

sample, $n=6$ /genotype) were run in triplicate on one 96-well plate for measurement of each steroid in one assay to ensure the comparability. The intra-assay coefficients of EIA are listed in Supplementary Table 2.

Statistical analysis

Values are presented as mean±s.d. A two-tailed independent Student's *t*-test was used to determine the differences between two groups. One-way ANOVA, followed by Tukey multiple comparison, was chosen for tests involving more than two groups. The relative frequency of sperm grades between the mutant and wild type fish was compared by chi-squared test. All analyses were performed using SPSS 22.0 (IBM). Thresholds corresponding to $P<0.05$ were used to identify statistically significant differences.

Results

Antibody verification and Cyp11c1 expression in developing gonad and head kidney

Unpurified and purified recombinant Cyp11c1, as well as total proteins extracted from 180 dah ovaries and testes, were analyzed by SDS-PAGE with Coomassie blue staining (Fig. 1A). The specificity of the anti-Cyp11c1 polyclonal antibody was confirmed by Western blot. Specific bands,

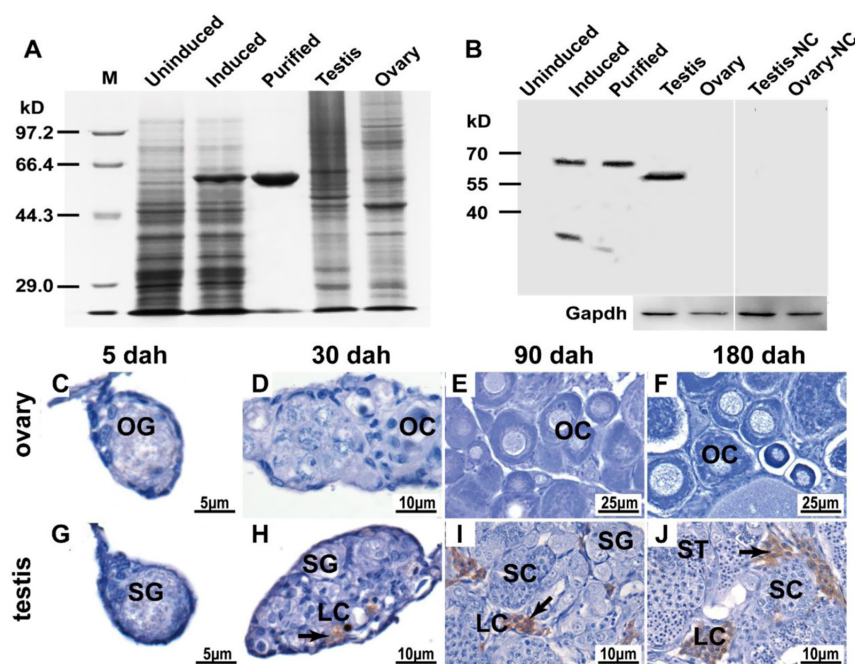


Figure 1

Cyp11c1 was specifically expressed in testicular Leydig cells. (A) SDS-PAGE and Coomassie blue staining of proteins from uninduced, IPTG-induced His-Cyp11c1-pCold I positive *E. coli*, together with purified His-Cyp11c1 protein and proteins from Nile tilapia testes and ovaries. (B) Specificity verification of anti-Cyp11c1 polyclonal antibody by Western blot. NC, negative control to show the staining in the ovaries and testes was diminished by addition of competing purified Cyp11c1 antigen. (C, D, E, f, G, H, I and J) Cellular localization of Cyp11c1 in testes and ovaries at 5, 30, 90 and 180 dah. Arrows, Cyp11c1-positive cells; OG, Oogonia; OC, Oocytes; SG, Spermatogonia; SC, Spermatocytes; ST, Spermatids; LC, Leydig cells; dah, days after hatching.

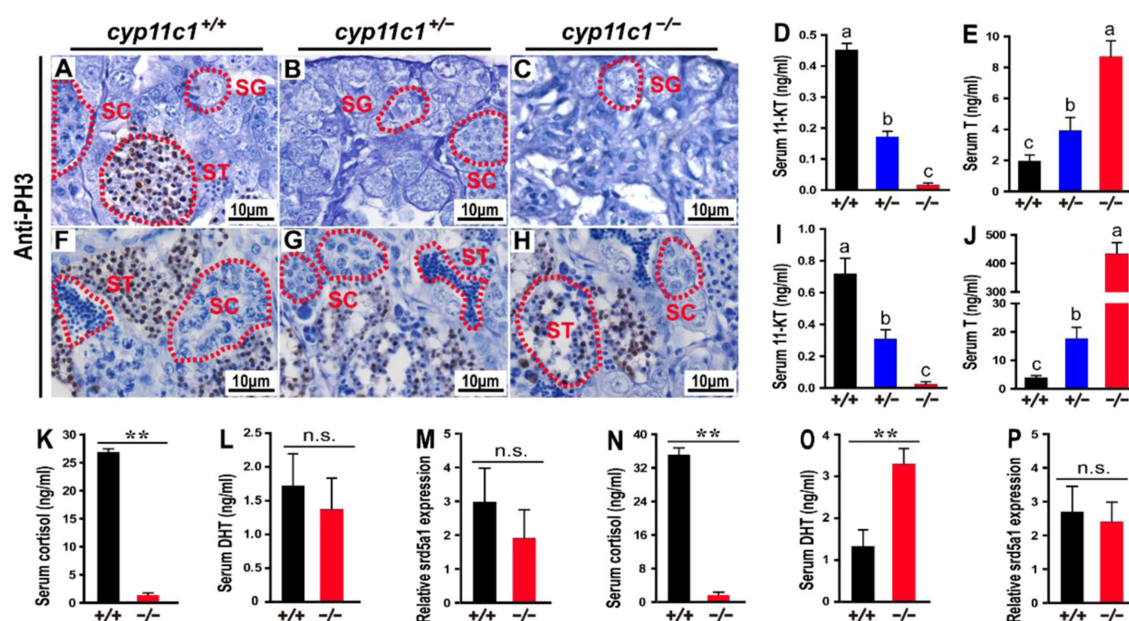


Figure 3

Mutation of *cyp11c1* resulted in delayed spermatogenesis. (A, B and C) PH3 staining of *cyp11c1*^{+/+} (A), *cyp11c1*^{+/-} (B) and *cyp11c1*^{-/-} (C) testis at 90 dah. (D and E) EIA measurement of serum 11-KT (D) and testosterone (T) (E) level of *cyp11c1*^{+/+}, *cyp11c1*^{+/-} and *cyp11c1*^{-/-} male fish at 90 dah. (F, G and H) PH3 staining of *cyp11c1*^{+/+} (F), *cyp11c1*^{+/-} (G) and *cyp11c1*^{-/-} (H) testis at 180 dah. (I and J) EIA measurement of serum 11-KT (I) and testosterone (T) (J) level of *cyp11c1*^{+/+}, *cyp11c1*^{+/-} and *cyp11c1*^{-/-} male fish at 180 dah. (K and L) Serum cortisol (K) and DHT (L) level of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} male fish at 90 dah. (M) Relative *srd5a1* mRNA expression of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} male fish by qPCR at 90 dah. (N and O) Serum cortisol (N) and DHT (O) level of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} male fish at 180 dah. (P) Relative *srd5a1* mRNA expression of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} male fish by qPCR at 180 dah. Red dotted lines outline spermatogonia (SG), spermatocytes (SC), spermatids (ST). The PH3 positive signal corresponds to brownish color. Values are presented as the mean $\pm$ s.d. ($n = 6$ /genotype). *** above the error bar indicate statistically significant differences at $P < 0.01$, as determined by two-tailed independent Student's *t*-test. Significant differences of serum 11-KT and testosterone level between *cyp11c1*^{+/+}, *cyp11c1*^{+/-} and *cyp11c1*^{-/-} group are indicated by different letters, as determined by one-way ANOVA followed by Tukey test for multiple comparisons ($P < 0.05$).

a delayed spermatogenesis, which might be caused by the deficiency of cortisol or 11-KT. The increased serum testosterone, thereafter, is likely a major contributor toward the restoration of spermatogenesis.

Delayed spermatogenesis could be rescued by exogenous 11-KT and testosterone, but not cortisol

To determine the major cause of delayed spermatogenesis at 90 dah, we analyzed *cyp11c1*^{-/-} males after administration of 11-KT and cortisol. We also compared the androgenic potency of testosterone with 11-KT in the initiation of spermatogenesis. Normal spermatogenesis with various stages of spermatogenic cells was observed in the testes after 11-KT and testosterone treatment (Fig. 4B, C, B' and C'), while spermatogenesis in the testes treated with cortisol was still aberrant (Fig. 4D and D'). To quantify these effects, we calculated cell numbers and relative proportions of spermatogenic cell types (Fig. 4E and F). Consistently, the testes with 11-KT and testosterone administration showed a relatively normal cell proportion (Fig. 4F). In addition, 11-KT displayed a higher androgenic potency

than testosterone, as reflected by a lower percentage of spermatogonia and spermatocytes, and higher percentage of spermatids (Fig. 4F). Collectively, these findings suggested that the aberrant spermatogenesis in *cyp11c1*^{-/-} at 90 dah was mainly caused by the deficiency of 11-KT, and not cortisol. 11-KT has a higher androgenic potency than testosterone.

Cyp11c1 ablation led to undersized testes and lower spermatogenic efficiency

The restored spermatogenesis of *cyp11c1*^{-/-} fish raised the question whether fish can develop a completely normal testis in the absence of 11-KT. We therefore analyzed testicular size, structure, spermatogenic efficiency and steroidogenic potency of *cyp11c1*^{-/-} fish at 360 dah. Despite the restored spermatogenesis, undersized testes with a relatively smaller efferent duct and disordered spermatogenic cysts were observed in *cyp11c1*^{-/-} fish (Fig. 5A, B, A', B' and E, F). In addition, *cyp11c1*^{-/-} fish showed a lower spermatogenic efficiency as indicated by the reduced semen volume (Fig. 5G). Besides, *cyp11c1*^{-/-} fish

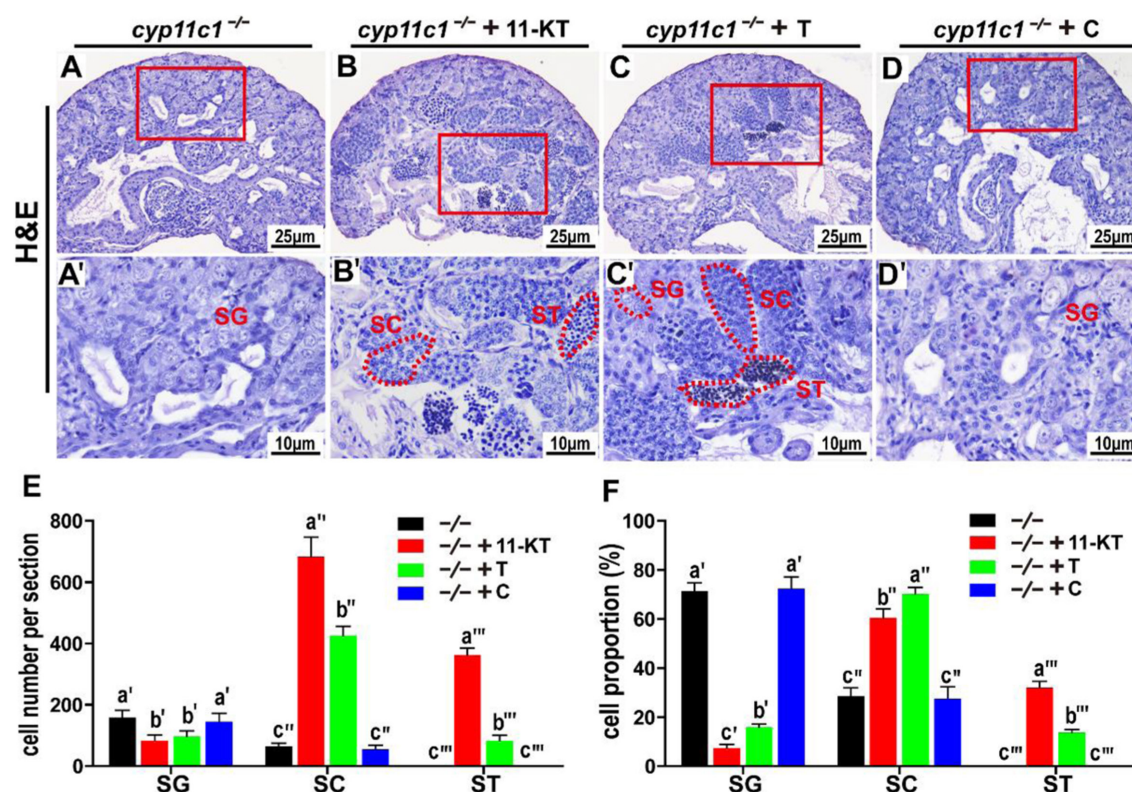


Figure 4

Delayed spermatogenesis in *cyp11c1*^{-/-} fish can be rescued by exogenous 11-KT and testosterone treatment. (A, B, C, D, A', B', C' and D') H&E staining of testes from fish at 90 dah after different treatment. Red dotted lines outline spermatogonia (SG), spermatocytes (SC), spermatids (ST). (E and F) Absolute cell number (E) and relative proportion (F) of spermatogenic cells from testes with different treatment. 11KT, 11-ketotestosterone; T, testosterone; C, cortisol. Values are presented as the mean ± s.d. (*n* = 5/treatment). Significant differences of SG, SC and ST between different treatment are indicated by different letters with ' ', ' ', ' ', respectively, as determined by one-way ANOVA followed by Tukey test for multiple comparisons (*P* < 0.05).

displayed a relatively reduced Leydig cell area, implying an impaired steroidogenic capacity (Fig. 5C, D, C', D' and H). These results demonstrated an abnormal morphology and compromised physiological function of *cyp11c1*^{-/-} testes. These defects were not detected in adult *cyp11c1*^{+/-} males (Supplementary Fig. 2). Considering the important role of gonadotropins in regulating steroidogenesis and testicular development, we further quantified the expression of *fshb*, *lhb* and their receptors, *fshr*, *lhr* by qPCR. To our surprise, the mRNA levels of these genes were upregulated in *cyp11c1*^{-/-} fish (Fig. 5I).

Effects of *cyp11c1* mutation on sperm quality

To get a more comprehensive assessment of the reproductive repertoire of *cyp11c1*^{-/-} males, semen from *cyp11c1*^{+/-} and *cyp11c1*^{-/-} fish was analyzed. Despite the reduced semen volume, *cyp11c1*^{-/-} fish showed normal sperm morphology and motility as indicated by the similar trajectory and sperm BCF, VSL, VCL (Fig. 6A, B, C,

D, E and F). To better evaluate sperm motility, the sperm of *cyp11c1*^{+/-} and *cyp11c1*^{-/-} fish with different motility was further graded with A (rapid motion forward), B (slow motion forward), C (no forward motion) and D (hyperslow or motionless). No significant differences were found in the frequency of this four sperm grades between *cyp11c1*^{+/-} and *cyp11c1*^{-/-} XY fish (Fig. 6G). These results revealed a normal sperm motility in *cyp11c1*^{-/-} XY fish. We further analyzed the fertilization ability of sperm. No significant differences were found between sperm from *cyp11c1*^{+/-} and *cyp11c1*^{-/-} fish (Fig. 6H). In conclusion, fish without 11-KT can produce a small amount of sperm with normal motility and fertilization ability.

Discussion

The impact of androgens and glucocorticoids on spermatogenesis has intrigued scientists for decades. *cyp11c1* is the key enzyme gene involved in the

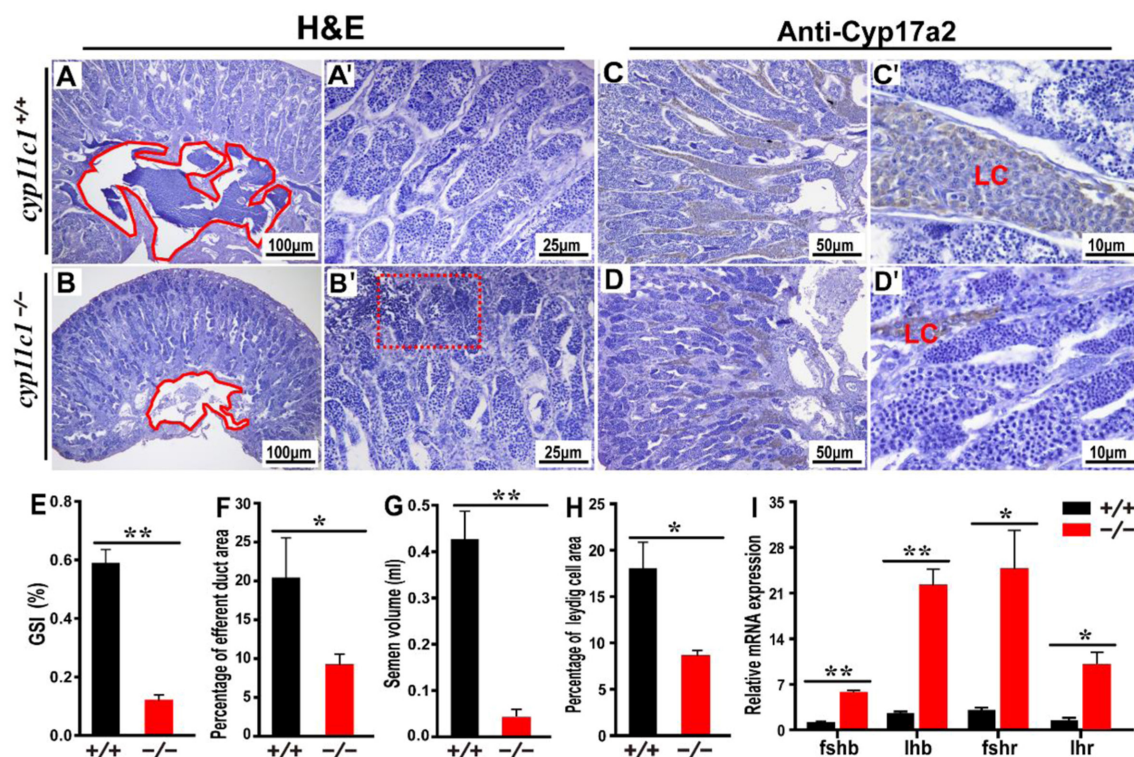


Figure 5

Mutation of *cyp11c1* resulted in disordered spermatogenic cysts and less semen. (A, B, A' and B') H&E staining of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} testis from fish at 360 dah. Red solid line outlines the efferent duct. Red dotted line highlights disordered spermatogenic cysts. (C, D, C' and D') Immunohistochemistry for the Leydig cell marker Cyp17a2 in *cyp11c1*^{+/+} and *cyp11c1*^{-/-} testis from fish at 360 dah. (E) GSI of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} male fish at 360 dah. (F) Percentage of efferent duct area per section at 360 dah. (G) Semen volume of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} fish. (H) Percentage of Leydig cell area per section. (I) Relative mRNA expression of *fshb*, *lhb*, *fshr*, *lhr* in *cyp11c1*^{+/+} and *cyp11c1*^{-/-} male at 360 dah. LC, Leydig cells. The positive signal corresponds to the brownish color. Values were presented as the mean $\pm$ s.d. ($n = 4$ /genotype). * and ** above the error bar indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, by two-tailed Student's *t*-test.

synthesis of 11-KT and cortisol, the major androgen and glucocorticoid in fish, respectively. In the present study, Cyp11c1 was found to be predominantly expressed in the testicular Leydig cells and head kidney interrenal cells

using specific antibodies. Through mutation of *cyp11c1* and a rescue experiment, we demonstrated that *cyp11c1* is important for the testicular development, especially for the initiation and proper proceeding of spermatogenesis.

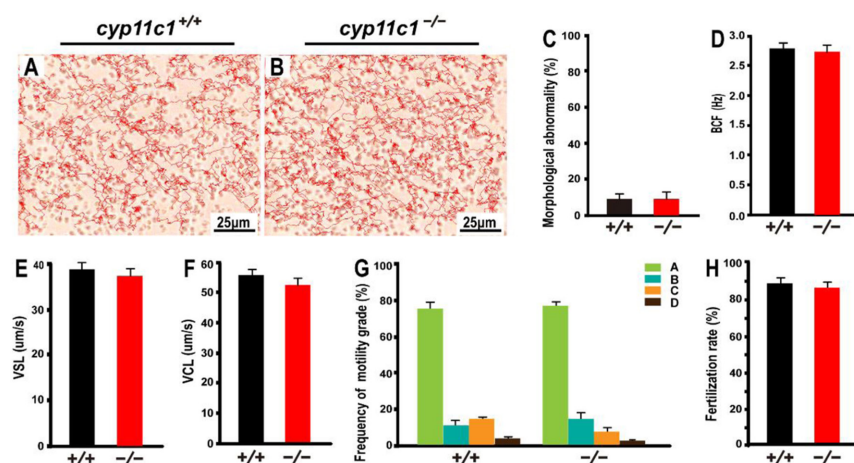


Figure 6

cyp11c1 mutation had no effect on sperm quality. (A and B) Sperm dynamic trajectory curve of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} fish (red line). (C, D, E and F) Evaluation of sperm morphology (C), BCF (D), VSL (E) and VCL (F). (G) Frequency of sperm motility grade. (H) Fertilization ability assessment of *cyp11c1*^{+/+} and *cyp11c1*^{-/-} sperm. BCF, beat cross frequency; VSL, straight line velocity; VCL, curvilinear velocity. Sperm motility grade A, rapid motion forward; B, slow motion forward; C, no forward motion; D, hyperslow or motionless. Values are presented as the mean $\pm$ s.d. ($n = 5$ /genotype). Differences between two groups were determined by two-tailed independent Student's *t*-test. The relative frequency of four sperm grades was estimated by Chi-squared test. No significant differences were observed between the *cyp11c1*^{+/+} and *cyp11c1*^{-/-} fish.

Cyp11c1 is expressed in Leydig cells and inter-renal cells in Nile tilapia

Sexually dimorphic expression of *cyp11c1* has been reported in a number of species (Jiang *et al.* 1996, Liu *et al.* 2000, 2009, Socorro *et al.* 2007). By *in situ* hybridization, *cyp11c1* was shown to be expressed in the testicular Leydig cells in rainbow trout and zebrafish (Kusakabe *et al.* 2002, Wang & Urban 2007). Similar expression was also reported in catfish by IHC (Rajakumar & Senthilkumaran 2015). Positive immunostaining of Cyp11c1/*cyp11c1* was also detected in spermatogonia in zebrafish (Caulier *et al.* 2015), sea bass (Viñas & Piferrer 2008) and Nile tilapia using a frog antibody (Zhang *et al.* 2010). In the present study, a tilapia-specific anti-Cyp11c1 polyclonal antibody was produced. By IHC, Cyp11c1 was found to be predominantly expressed in the testicular Leydig cells at 30, 90, 180 dah and head kidney inter-renal cells at 1, 30, 90, 180 dah. No significant expression was observed in ovaries at any developmental stage, or in testes at 5 dah. No specific immunostaining was detected in spermatogonia in the present study. Previously, two alternatively spliced *cyp11c1* isoforms were detected in testis and head kidney by RT-PCR and Northern blot (Zhang *et al.* 2010). However, only one band, corresponding to the longer isoform, was detected in the present study. This antibody recognizes both isoforms as the whole sequence of Cyp11c1 was used as antigen. We thus speculated that the short isoform was not translated into protein in tilapia. Cyp11c1 was also shown to be expressed in ovary in some fish species, such as honeycomb grouper and anemonefish (Alam *et al.* 2005, Miura *et al.* 2008). In this study, no Cyp11c1 expression was detected in tilapia ovary by IHC and Western blot, which is consistent with the results reported previously by our group (Zhang *et al.* 2010). Therefore, species differences in tissue expression of Cyp11c1 may exist in fishes.

11-KT is critical for the initiation of spermatogenesis

Androgens are necessary for spermatogenesis in mammals (Smith & Walker 2014). In fish, the impact of androgens on spermatogenesis remains an ongoing debate. Unlike in mammals, 11-KT instead of testosterone is generally considered to be the major androgen in teleost fish (Borg 1994). On the one hand, it is well-documented that 11-KT is tightly associated with the process of spermatogenesis (Mayer *et al.* 1990, Kusakabe *et al.* 2002, Rajakumar & Senthilkumaran 2015). 11-KT has been shown to induce all stages of spermatogenesis in Japanese eel

in vitro (Miura *et al.* 1991). Exogenous 11-KT supplement promotes spermatogenesis in goldfish and Japanese huchen by inducing the initiation of spermatogonial proliferation (Kobayashi *et al.* 1991, Amer *et al.* 2001). In addition, African catfish treated with 11-KT displayed precocious spermatogonial proliferation and meiosis (Cavaco *et al.* 1998). Moreover, in tilapia, the initiation of spermatogenesis was reported to be significantly promoted by 11-KT in the presence of insulin-like growth factor 1 (IGF1) or human chorionic gonadotropin (hCG) (Tokalov & Gutzeit 2005). These results suggest that androgens are important for fish spermatogenesis. On the other hand, defective synthesis of androgens, caused by *cyp17a1* deletion, did not result in spermatogenesis defects in medaka and zebrafish (Sato *et al.* 2008, Zhai *et al.* 2018). Zebrafish treated with flutamide (a specific antagonist of the androgen receptor) or subject to a complete shutdown of androgen signaling by AR knockout, still produce a small amount of viable sperm and are fertile (Yin *et al.* 2017, Tang *et al.* 2018). These results support the argument that androgens are not indispensable for spermatogenesis.

In the present study, loss of *cyp11c1* resulted in sharply decreased serum cortisol and 11-KT. Meanwhile, a delayed spermatogenesis with only spermatogonia was observed in *cyp11c1*^{-/-} testes at 90 dah, implying a possible role of cortisol or/and 11-KT in the initiation of spermatogenesis. The fact that delayed spermatogenesis could be rescued by 11-KT but not cortisol in *cyp11c1*^{-/-} male suggests that 11-KT but not cortisol (at least not directly) contribute to the aberrant initiation of spermatogenesis. Overall, although the actions of other androgens in spermatogenesis could not be excluded in *cyp11c1*^{-/-} tilapia, the role of 11-KT for the onset of spermatogenesis is noteworthy.

11-KT is the most efficient androgen in teleost fish

The androgenic effect of 11-KT was firstly identified in the male sockeye salmon (Idler *et al.* 1961). Since then, this steroid has been shown to be synthesized in the testis following gonadotropins stimulation in various teleost fish, resulting in high serum levels during spermatogenesis (Billard *et al.* 2017). Although the action of 11-KT in spermatogenesis has been proven in different species (Kobayashi *et al.* 1991, Miura *et al.* 1991, Cavaco *et al.* 1998, Amer *et al.* 2001), whether 11-KT is indispensable for fish spermatogenesis remains to be seen. In the present study, 11-KT synthesis was completely blocked by mutation of *cyp11c1*. Fish without 11-KT showed a delayed spermatogenesis, undersized testis but normal sperm motility. Here, two points need to be highlighted.

First, the restored spermatogenesis in *cyp11c1*^{-/-} male at 180 dah might result from the compensation of other androgenic steroids in spermatogenesis. In this study, increased serum testosterone was detected both at 90 and 180 dah. Nevertheless, despite the elevated serum testosterone level, delayed spermatogenesis was still observed, indicating a higher potency of 11-KT than testosterone in the proper initiation of fish spermatogenesis. Furthermore, significantly reduced semen volume was observed in the *cyp11c1*^{-/-} male, demonstrating a higher potency of 11-KT than testosterone in the promotion of fish spermatogenesis. DHT was generally considered to be produced only in tetrapods (Martyniuk *et al.* 2013). However, this steroid recently has been suggested to play an important role in some fish species (Margiotta-Casaluci & Sumpter 2011, Margiotta-Casaluci *et al.* 2013, González *et al.* 2015, García-García *et al.* 2017). In the present study, increased serum DHT was detected in *cyp11c1*^{-/-} tilapia at 180 dah. Even though the elevated DHT level might be the consequence of testosterone accumulation, the possible role of DHT in the restoration of delayed spermatogenesis could not be excluded. This is also consistent with our previous study that showed some precursors of the 11-KT synthesis pathway, such as androstenedione, testosterone and DHT, could also act as androgens in tilapia (Shi *et al.* 2017). The exact role of DHT in fish reproduction needs to be further explored.

Second, *cyp11c1*^{-/-} male tilapia displayed undersized testes with smaller efferent duct and disordered spermatogenic cysts. This is similar to AR-knockout zebrafish (Tang *et al.* 2018). The gonadotropins, FSH and LH are the most important pituitary hormones regulating testicular development (Koskenniemi *et al.* 2017). We thus

wondered whether the defective testes in *cyp11c1*^{-/-} fish were caused by gonadotropin insufficiency considering the highly elevated serum testosterone. Contrary to our expectations, increased expression of *fshb*, *lhb* and their receptors, *fshr*, *lhr*, was detected, indicating that the undersized testis in *cyp11c1*^{-/-} male fish was a direct effect of 11-KT deficiency, instead of a negative feedback of increased testosterone to gonadotropin synthesis. These results indicate an incomparable role of 11-KT in fish testicular development. However, spermatogenesis was self-restored and small amounts of viable sperm were produced in *cyp11c1*^{-/-} male tilapia. Taken together, these results demonstrated that 11-KT is the most efficient androgen for fish testicular development, especially for the initiation and progression of spermatogenesis.

The action of cortisol in spermatogenesis might be mediated by 11-KT

Cortisol is the main glucocorticoid in teleost fish. The crosstalk between the cortisol and androgen pathways in temperature induced masculinization has been reported in several species (Hayashi *et al.* 2010, Fernandino *et al.* 2012). Whether the action of cortisol in testicular development and spermatogenesis is independent of androgens or not remains unclear. On the one hand, in Japanese eel, optimal levels of cortisol have been demonstrated to induce spermatogonial mitosis by increasing 11-KT production (Ozaki *et al.* 2006). On the other hand, in common carp, restoration of serum 11-KT could not rescue the cortisol-induced retardation of testicular growth and the first wave of spermatogenesis (Consten *et al.* 2002), indicating an androgen-independent action of cortisol. In this study,

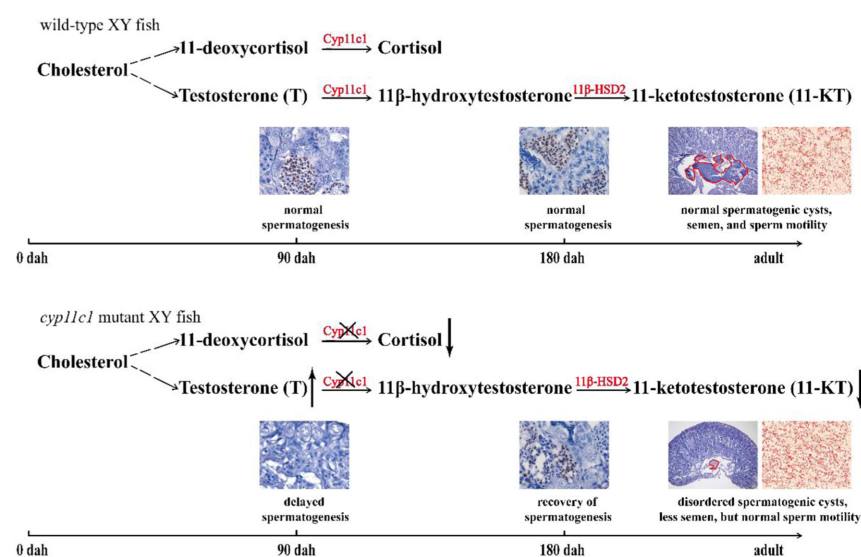


Figure 7

Schematic representation of the role of *cyp11c1* on spermatogenesis in tilapia. *cyp11c1* mutant fish displayed delayed spermatogenesis, disordered spermatogenic cysts, less semen, but normal sperm motility due to the potential compensatory effect of testosterone in the absence of 11-KT.

exogenous cortisol supplement failed to rescue the delayed spermatogenesis in *cyp11c1*^{-/-} fish, suggesting the action of cortisol in spermatogenesis might be 11-KT dependent. Our results could further serve as a new evidence for the crosstalk between cortisol and 11-KT.

In conclusion, loss of *cyp11c1* in male tilapia caused delayed spermatogenesis at 90 dah, which could be rescued by exogenous 11-KT or testosterone, but not cortisol, indicating an important role of 11-KT in the initiation of fish spermatogenesis. Notably, spermatogenesis was restored spontaneously at 180 dah, probably due to compensation by other androgenic steroids, such as testosterone or DHT. Furthermore, *cyp11c1* ablation led to undersized testis with smaller efferent duct, disordered spermatogenic cysts and significantly reduced semen with viable sperm in adult tilapia (Fig. 7). These results suggest that 11-KT is the most efficient androgen in tilapia.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0438>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Deshou Wang and Minghui Li conceived and designed the experiments; Qiaoyuan Zheng, Hesheng Xiao, Hongjuan Shi, Tingru Wang, Minghui Li, Lina Sun and Wenjing Tao performed the experiments; Deshou Wang, Qiaoyuan Zheng and Hesheng Xiao analyzed the data, interpreted the results and drafted the manuscript; Thomas D Kocher critically edited the manuscript. All authors read and approved the final manuscript.

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