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Investigating the molecular mechanisms of oocyte maturation and ovulation in Nile tilapia: A focus on the steroidogenic enzyme Cyp17a2

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ABSTRACT

Previous research has highlighted the significant role of progestins and glucocorticoids in fish oocyte maturation and ovulation. To clarify the molecular mechanisms underlying these processes, comprehensive investigations were conducted using a *cyp17a2* mutant Nile tilapia (*Oreochromis niloticus*) model. Analysis revealed pronounced Cyp17a2 expression in ovarian somatic cells of the tilapia. Female *cyp17a2*-deficient mutants exhibited markedly reduced levels of 17,20 β -dihydroxy-4-pregnen-3-one (DHP) and cortisol/cortisone, leading to delayed meiotic initiation and impaired oocyte maturation and spawning. Notably, supplementation with human chorionic gonadotrophin (hCG), DHP, and cortisol effectively induced germinal vesicle breakdown (GVBD) and facilitated oocyte release with follicular cell layers in *cyp17a2*^{-/-} females. Additionally, *cyp17a2*^{-/-} and rescued *cyp17a2*^{-/-} females showed elevated transcription of steroidogenic enzymes involved in 17 β -estradiol (E2) production compared to spawning wild-type females. Moreover, the reduction in Akt phosphorylation observed in *cyp17a2*-deficient females and upon inhibitor treatment impaired hCG-induced oocyte maturation. Conversely, activation of the phosphoinositide 3-kinase/protein kinase B (PI3K-Akt) signaling pathway partially rescued the oocyte maturation impairment caused by *cyp17a2* mutation. Overall, these findings provide functional evidence supporting the critical role of Cyp17a2 in DHP and cortisol biosynthesis, which, in turn, facilitates oocyte maturation and ovulation through activation of the PI3K-Akt signaling pathway in fish.

Keywords: DHP; Cortisol; Reproductive process; PI3K-Akt; Tilapia

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INTRODUCTION

Oocyte maturation and ovulation constitute two coordinated reproductive processes essential for the production and timely release of high-quality oocytes (Robker et al., 2018). These processes are tightly regulated by a complex network of endocrine and intrafollicular paracrine signals. Notably, luteinizing hormone (LH) release stimulates the production of maturation-inducing hormone (MIH) within the follicular cells through the brain-pituitary-gonadal axis, thereby promoting oocyte maturation and ovulation in fish (Nagahama & Yamashita, 2008; Shao et al., 2021). Among teleosts, 17,20 β -dihydroxy-4-pregnen-3-one (DHP) and 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S) have been identified as potent MIHs, effectively inducing germinal vesicle breakdown (GVBD) during oocyte maturation.

The endocrine regulation of oocyte maturation and ovulation in fish has been extensively investigated. Immediately prior to spawning, fish ovarian follicles undergo a significant shift in the steroidogenic pathway, from 17 β -estradiol (E2) production during oocyte growth to DHP synthesis during oocyte maturation. This transition is closely aligned with the steroidogenic enzyme expression profiles observed throughout the reproductive cycle (Nagahama et al., 1993). We previously found that high cytochrome P450, family 19 subfamily A polypeptide 1a (*cyp19a1a*), and cytochrome P450 family 17 subfamily A member 1 (*cyp17a1*) expression in follicular cells is correlated with E2 production during the oocyte growth phase. As the oocyte advances to the maturation stage, there is a marked reduction in *cyp19a1a* and *cyp17a1* expression, accompanied by a rapid increase in *cyp17a2* expression—a paralog of *cyp17a1*—within the granulosa cells, suggesting a potential role for Cyp17a2 in fish

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oocyte maturation (Zhou et al., 2007a).

Glucocorticosteroids, synthesized through the hydroxylase activity of Cyp17a in the interrenal cells of the head kidney, play an essential role in female reproductive processes. In mammals, physiological stress can have an inhibitory effect on reproductive functions, leading to follicular atresia, delays in oocyte maturation, and disruptions in ovulation (Kala & Nivsarkar, 2016; Zhang et al., 2011). Additionally, cortisol levels increase in ovulatory follicles in response to an LH surge or human chorionic gonadotropin (hCG) administration in the human ovary, suggesting that cortisol may act as an anti-inflammatory factor within these follicles, contributing to ovulation and corpus luteum formation by binding to glucocorticoid receptors (GRs) (Hillier & Tetsuka, 1998; Jeon et al., 2023; Kushnir et al., 2009). In fish, accumulating evidence has demonstrated the dynamic role of cortisol during oogenesis, oocyte maturation, and ovulation, highlighting its importance in reproductive processes (Sopinka et al., 2017; Xiao et al., 2022). Inhibition of 11 β -hydroxylase, either through inhibitor intervention or genetic mutation, has been shown to significantly repress the ovulatory response to gonadotropin and induce follicular cell apoptosis (Sundararaj & Goswami, 1966; Xiao et al., 2022). The pronounced hydroxylase activity of *cyp17a2*, along with its prominent expression in the interrenal cells of the head kidney in both medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*) relative to *cyp17a1*, supports the involvement of Cyp17a2 in cortisol biosynthesis (Zhou et al., 2007a, 2007b). These findings suggest that Cyp17a2 is central to steroidogenesis, contributing to both DHP and cortisol production, and may play a crucial role in the regulation of oocyte maturation and ovulation. Thus, further functional studies on Cyp17a2 are essential to unravel the molecular mechanisms and regulatory networks governing these reproductive processes.

The serine/threonine protein kinase Akt is an integral component of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, which regulates various cellular processes, including cell cycle progression, cellular growth, and embryogenesis (Verma et al., 2023). Gonadotropins have been shown to promote Akt phosphorylation by binding to their receptors, thereby activating the PI3K/Akt signaling pathway (Law & Hunzicker-Dunn, 2016; Tai et al., 2009). Once activated, the PI3K/Akt signaling pathway stimulates several downstream substrates that regulate key cellular processes, such as nuclear envelope breakdown, centrosome maturation, spindle assembly, chromosome segregation, and cytokinesis (Cecconi et al., 2010; Hoshino & Sato, 2008; Kalous et al., 2006). Additionally, evidence suggests that progesterone functions upstream of the PI3K/Akt signaling pathway, potentially inducing Akt phosphorylation (Liu et al., 2023; Sagare-Patil et al., 2013).

Nile tilapia, a globally important aquaculture fish species, possesses several advantageous traits, including a well-characterized reproductive cycle, high fecundity, amenability to *in vitro* fertilization, and genetic manipulability, making it an ideal model for studying the molecular mechanisms underlying oocyte maturation and ovulation in fish (Nagahama & Yamashita, 2008; Senthilkumaran et al., 2002). The *cyp17a2* gene, a homolog of *cyp17a1*, retains 17 α -hydroxylase activity like Cyp17a1 but lacks C17-20 lyase activity (Lai et al., 2022; Zhou et al., 2007a, 2007b). In tilapia, *cyp17a2* expression persists in theca cells throughout the spawning cycle, with a sharp up-regulation observed in granulosa cells during oocyte

maturation (Zhou et al., 2007a). Homozygous *cyp17a2* mutations in male tilapia are associated with significant decreases in both cortisol and DHP production and impaired spermatogenesis (Yang et al., 2022). Therefore, we hypothesize that *cyp17a2* is crucial for the biosynthesis of cortisol and DHP, which subsequently promote oocyte maturation and ovulation through the activation of the downstream PI3K-Akt signaling pathway.

MATERIALS AND METHODS

Animals

Nile tilapia fish were acclimatized in recirculating, aerated freshwater tanks maintained at 26°C and exposed to a natural photoperiod. In the group domesticated in our laboratory, sexually mature wild-type XX females exhibited a distinct 14-day spawning cycle accompanied by nesting behavior prior to spawning. The *cyp17a2* mutant fish were generated by clustered regularly interspaced short palindromic repeats/CRISPR-associated system (CRISPR/Cas9) technology (Yang et al., 2022). All animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals approved by the Committee of Laboratory Animal Experimentation of Southwest University, China (No. IACUC-20181015-12, 15 October 2018).

Sampling and histological examination

The body weight and age of each sampled fish were recorded prior to histological analysis. Both ovaries from each fish were excised and weighed, with the gonadosomatic index (GSI; ratio between ovary and body weight) then calculated. After sampling, the ovaries were fixed in Bouin's solution for 12 h at room temperature, then dehydrated and embedded in paraffin. Subsequently, the tissue blocks were sectioned (5 μ m slices) and stained with hematoxylin and eosin (H&E) for histological examination.

Immunohistochemical (IHC) analysis

IHC analyses were performed to detect the cellular localization of Cyp17a2 in the tilapia ovaries at different stages and to assess the impacts of *cyp17a2* mutation on oogenesis, oocyte maturation, and ovulation. Ovaries from both wild-type and *cyp17a2*^{-/-} XX fish were dissected at different developmental stages, fixed in Bouin's solution for 12 h at room temperature, dehydrated, and embedded in paraffin. The tissue blocks were then sectioned (5 μ m) and treated with blocking solution (5% bovine serum albumin (BSA) diluted in 1 $\times$ phosphate-buffered saline (PBS), Sangon Biotech, China). Subsequently, the sections were incubated with primary antibodies against -Cyp17a2 (1:1 000 dilution, AB_3099676), -Vasa (germ cell marker, 1:1 000 dilution, AB_289326), -Dmrt1 (Doublesex and mab-3 related transcription Factor 1, male specific Sertoli cell marker, 1:1 000 dilution, AB_2650456), -Gsdf (gonadal soma-derived factor, male-biased Sertoli cell marker, 1:1 000 dilution, AB_2650457), and -Cyp19a1a (follicular cell marker, 1:1 000 dilution, AB_2629226) overnight at 4°C, then rinsed with 1 $\times$ PBS five times (5 min each). As a negative control, the primary antibodies were substituted with normal rabbit serum. The slides were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1 000 dilution, AB_228341, Invitrogen, USA) at room temperature for 1 h, followed by three successive rinses with 1 $\times$ PBS (5 min each). Immunoreactive signals were visualized using

diaminobenzidine tetrachloride (DAB, Sigma, Germany) as the substrate, followed by counterstaining with hematoxylin. Images were captured using an Olympus BX5 light microscope (Olympus, Japan).

In vitro ovarian culture

An *in vitro* oocyte maturation assay was conducted in tilapia to explore the essential role of DHP and changes in PI3K-Akt signals in hCG-induced oocyte maturation, following previously described methods for zebrafish (*Danio rerio*) (Tokumoto et al., 2011). Ovaries from *cyp17a2^{+/+}* and *cyp17a2^{-/-}* XX tilapia were isolated from sacrificed females at 360 days after hatching (dah), rinsed with fresh tilapia Ringer's solution (140 mmol/L NaCl, 10 mmol/L NaHCO₃, 5 mmol/L KCl, 1 mmol/L CaCl₂, 2 mmol/L NaHPO₄, 1 mmol/L CaCl₂, and 5.5 mmol/L glucose, pH 7.2), and dissected into fragments containing 8–15 oocytes using fine forceps and surgical scissors. Immature, fully grown oocytes were cultured *in vitro* by incubating the ovarian fragments in 7 mL of M119 medium at 28°C. The concentrations of reagents used in the *in vitro* culture include 50 IU/mL hCG (Ningbo Second Hormone Factory, China) (Mukherjee et al., 2006), 1 µg/mL DHP (Cayman, USA) (Selman et al., 1994), 100 µmol/L LY294002 (MCE, USA) (Jiang et al., 2010), and 30 µmol/L 740 Y-P (MCE, USA) (Feng et al., 2018).

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

Gene expression at the transcript level was compared between wild-type XX and *cyp17a2^{-/-}* fish using RT-qPCR. Ovarian tissue and follicular cells were collected from *cyp17a2^{+/+}* XX (*n*=3) and *cyp17a2^{-/-}* XX (*n*=3) fish. Total RNA (1.0 µg) was extracted and reverse transcribed with a Prime Script RT Master Mix Perfect Real Time Kit (Takara, Japan) following the manufacturer's guidelines. Subsequently, RT-qPCR was performed in a 20 µL reaction volume on an ABI-7500 Fast Real-Time PCR system (Applied Biosystems, USA). Each reaction included 10 µL of 2×SYBR Premix ExTaq (Takara, Japan), 2 µL of diluted cDNA (or PCR-grade water as a negative control), 6 µL of PCR-grade water, and 1 µL of each 10 µmol/L primer. The PCR reaction conditions included denaturation at 95°C for 5 min; followed by 40 amplification cycles at 95°C for 15 s and 60°C for 30 s. Melting curves were assessed using dissociation protocols, and the relative abundance of target genes were evaluated using the ($R=2^{-\Delta\Delta Ct}$) formula (Livak & Schmittgen, 2001), with β -actin serving as an internal control to verify reaction efficiency. The primer sequences used for PCR are listed in Supplementary Table S1.

Follicle counting

The ovaries of 90-dah wild-type (*n*=6) and *cyp17a2^{-/-}* (*n*=6) fish were fixed, embedded in paraffin, and sectioned (5 µm slices). Follicles from the ovarian sections were counted for analysis according to previously described methods (Fang et al., 2018). Histological classification of the oocytes was conducted in accordance with previous protocols (Liu et al., 2020).

Measurement of serum steroid hormone levels via enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Blood samples were collected from the caudal veins of *cyp17a2^{+/+}* XX and *cyp17a2^{-/-}* XX fish following anesthesia (250 mg/L, MS-222, Sigma, USA) and kept at room

temperature for 1 h. Following centrifugation at 664 ×*g* for 10 min at room temperature, the supernatant was transferred to a clean centrifuge tube and centrifuged at 10 625 ×*g* for 10 min at 4°C and stored at -80°C until further analysis. Serum concentrations of DHP, cortisol, and E2 were measured at 90 (*n*=4 fish/genotype) or 180 dah (*n*=5 fish/genotype) using ELISA kits (Cayman, USA) according to the manufacturer's instructions. This approach has previously been employed for serum hormone quantification in tilapia (Fang et al., 2018; Yang et al., 2021). Intra-assay coefficients of variation ranged from 5.18% to 14.76%, and inter-assay coefficients of variation ranged from 11.94% to 25.71%. Comprehensive profiling of serum steroid levels in adult *cyp17a2^{+/+}* XX and *cyp17a2^{-/-}* XX fish (*n*=3 fish/genotype) was conducted using the AB Sciex QTRAP® 6500 LC-MS/MS system (AB Sciex, USA) by Biomarker Technologies. All steroid information is listed in Supplementary Table S2.

Determination of GVBD

To determine the effect of *cyp17a2* deficiency on oocyte maturation, organic reagents (ethanol, formalin, and acetic acid) were used to detect the GVBD ratio, as described in previous study (Chattoraj et al., 2005). Briefly, oocytes were collected from *cyp17a2^{+/+}* XX and *cyp17a2^{-/-}* XX fish and immersed in a clearing solution (ethanol:formalin:acetic acid, 6:3:1, volumetrically) to facilitate examination under a stereoscopic microscope (Leica, M205 FA, Germany) equipped with in-built photo-micrographic attachments and an image analysis device (Chattoraj et al., 2005).

Rescue experiments

At 30 dah, *cyp17a2^{-/-}* XX fish were allocated into control, cortisol treatment, and DHP treatment groups. The fish in the cortisol treatment group were fed a diet sprayed with 95% ethanol containing cortisol (Yuanye, China) at a concentration of 200 µg/g feed (Zheng et al., 2020). The fish in the DHP treatment were housed in a 20 L tank with 10 nmol/L DHP (Cayman, USA) (Tokumoto et al., 2011). Gonadal samples from all groups were collected at 90 dah for histological analysis to assess gonadal development.

To assess the impacts of *cyp17a2* gene mutation on oocyte maturation and ovulation, sexually mature *cyp17a2^{-/-}* XX fish were allocated into control, 17 α -hydroxyprogesterone (17 α -OHP)+hCG treatment, DHP+hCG treatment, and DHP+hCG+cortisol treatment groups. The treated fish were administered a diet sprayed with 95% ethanol containing 17 α -OHP (TCI, Japan) at a concentration of 400 µg/g feed or cortisol (Yuanye, China) at a concentration of 200 µg/g feed, as previously documented (Lee et al., 1999; Zheng et al., 2020). Due to the chemical properties and dosages of the reagents, DHP (Cayman, USA) and hCG (Ningbo Second Hormone Factory, China) were administered via intraperitoneal injection once a week, as previously described (Yueh & Chang, 1997). The control fish were fed a diet sprayed with 95% ethanol only.

Immunofluorescence (IF) staining of oocytes

Oocytes were collected from *cyp17a2^{+/+}* XX individuals that had not undergone spawning and from steroid-rescued *cyp17a2^{-/-}* XX individuals. These oocytes were fixed in 4% paraformaldehyde (PFA, Biosharp, China) in PBS at 4°C overnight with gentle shaking, then rinsed with PBS three times, permeabilized with 3‰ Triton X-100 (Beyotime, China) in PBS for 20 min, and blocked with 5% BSA in PBS at room

temperature for 30 min. Subsequently, the oocytes were incubated with primary antibody against Cyp19a1a (1:1 000 dilution) at 4°C overnight, followed by three washes with PBS and subsequent incubation at 4°C overnight with goat anti-rabbit antibody Alexa Fluor 488-conjugated secondary antibody (Invitrogen, USA) diluted at a 1:1000 ratio in blocking solution. The nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) and visualized using an FV3000 confocal microscope (Olympus, Japan) to capture the fluorescence signals.

Follicular cell layer collection

Follicular cells surrounding oocytes were physically separated from three preovulatory wild-type XX individuals (CTRL), three *cyp17a2*^{-/-} XX female mutants (*cyp17a2*^{-/-}), three spawned wild-type XX females (CTRL-S), and three postovulatory *cyp17a2*^{-/-} XX females rescued by steroids (*cyp17a2*^{-/-}-R). Procedures were carried out according to methods previously used in zebrafish (Liu et al., 2017). The follicular cells were collected into a 1.5 mL microcentrifuge tube and immediately homogenized in RNAiso Plus solution (Takara, Japan). Subsequently, RNA extraction and reverse transcription were conducted for RT-qPCR analysis, as described above.

Western blotting

Total protein was extracted from ovarian fragments of *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish, incubated with or without the reagents mentioned above, and diluted to a final concentration of 20 mg/mL. After adding loading buffer (Beyotime, China), proteins were denatured by heat treatment, then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, EpiZyme, China) and transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham, England). The membranes were then blocked with 5% non-fat milk powder (Sangon Biotech, China) in Tris-buffered saline with Tween (TBST) (20 mmol/L Tris-HCL pH7.5, 150 mmol/L NaCl, 0.1% Tween 20) for 1 hour at room temperature, followed by primary antibodies against -Akt (#9772S, Cell Signaling, USA) and -p-Akt (#4060S, Cell Signaling, USA) incubation overnight at 4°C. The HRP-labeled secondary antibodies (AB_228341, Invitrogen, USA) were then incubated for 1 h at 37°C. Finally, positive immunoreactivity was identified using a BeyoECL Plus Kit (Beyotime, China) and visualized on a Fusion FX7 imaging system (Vilber Lourmat, France). Densitometry was quantified using Fusion-CAPT software and normalized using α -tubulin (AB_2619646, Cell Signaling, USA) as the reference protein.

Data analysis

Data from RT-qPCR, GSI, ELISA, follicle counting, and western blot densitometry are presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using Student's *t*-test for two-group comparisons, and one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test for multiple comparisons using SPSS v.16.0. Statistical significance was defined at *P* < 0.05.

RESULTS

Cellular localization of Cyp17a2 in the tilapia ovary and response to hCG induction *in vitro*

Cyp17a2-specific polyclonal antibodies were used to characterize the cellular localization of Cyp17a2 in the tilapia ovary at different developmental stages. Positive signals for

Cyp17a2 were initially detected in the cells surrounding the germ cells and the follicular cells within the gonads of XX fish at 30 dah (Figure 1A). Moreover, RT-qPCR analysis revealed an up-regulation of genes involved in the biosynthesis of DHP and cortisol (*cyp17a2* and *hsd11b2*), as well as nuclear receptors (*pgr*, *gr1*, and *gr2*) for both hormones, in the ovaries of wild-type XX fish treated with hCG *in vitro* (Figure 1B).

Cyp17a2 mutation arrests meiotic initiation during early stages of ovarian development

Histological analyses revealed the presence of oocytes at phases I and II in the ovaries of *cyp17a2*^{+/+} XX fish, as well as significant oogonia proliferation and phase I and II oocyte decrease in the ovaries of *cyp17a2*^{-/-} XX fish at 90 dah (Figure 2A). Vasa-positive signals, a marker of germ cells, demonstrated a significant delay in meiotic initiation in the gonads of *cyp17a2*^{-/-} XX fish at 90 dah (Figure 2B). Comparative cell counts confirmed an increased number of oogonia and a reduced number of phase I and II oocytes in *cyp17a2*^{-/-} XX fish relative to *cyp17a2*^{+/+} XX fish at 90 dah (Figure 2C). In addition, the expression levels of *piwil* (germ cell marker), *sycp3*, *spo11*, and *aldh1a2* (meiosis-related markers) were notably reduced in *cyp17a2*^{-/-} XX fish compared to *cyp17a2*^{+/+} XX fish at 90 dah (Figure 2D).

ELISA results showed no significant differences in E2 biosynthesis between the wild-type and *cyp17a2*^{-/-} XX fish. However, DHP and cortisol synthesis levels were markedly reduced in *cyp17a2*^{-/-} XX fish compared to wild-type XX females at 90 dah (Figure 2E–G). Notably, exogenous DHP partially rescued the meiotic initiation delay caused by *cyp17a2* deficiency in female tilapia, while cortisol treatment had no significant effect, as revealed by H&E and IF staining for Vasa (Figure 2H; Supplementary Figure S1). Further examination of the *cyp17a2* mutation effects on sex differentiation revealed positive immunoreactivity for Cyp19a1a and Gsdf, but not Dmrt1, in the gonads of *cyp17a2*^{-/-} XX fish at 90 dah (Supplementary Figure S2A). Additionally, RT-qPCR analyses showed no significant differences in the gonadal transcription levels of *cyp17a1* and *cyp19a1a* between *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish (Supplementary Figure S2B, C). Collectively, these results suggest that Cyp17a2 is crucial for initiating meiosis in female tilapia but does not play a role in sex differentiation.

Cyp17a2 plays a crucial role in progesterin production and female fertility

Under controlled laboratory conditions, adult wild-type females typically spawned twice a month and exhibited nesting behaviors prior to spawning (Supplementary Video S1). In contrast, *cyp17a2* mutant females displayed no nesting behaviors and were unable to spawn (Figure 3D; Supplementary Video S2). Anatomical analysis revealed a higher prevalence of degenerated oocytes in *cyp17a2*^{-/-} XX females compared to *cyp17a2*^{+/+} XX females at 360 dah (Figure 3A). Despite this, no significant differences in ovarian morphology or GSI were identified between the two groups (Figure 3B, C). Based on ELISA, the serum concentrations of DHP and cortisol were significantly reduced in *cyp17a2*^{-/-} XX fish, while E2 biosynthesis remained unaffected when compared to that of *cyp17a2*^{+/+} XX fish (Figure 3E–G).

Effects of *cyp17a2* gene mutation on oocyte maturation and ovulation

The effects of *cyp17a2* gene mutation on oocyte maturation

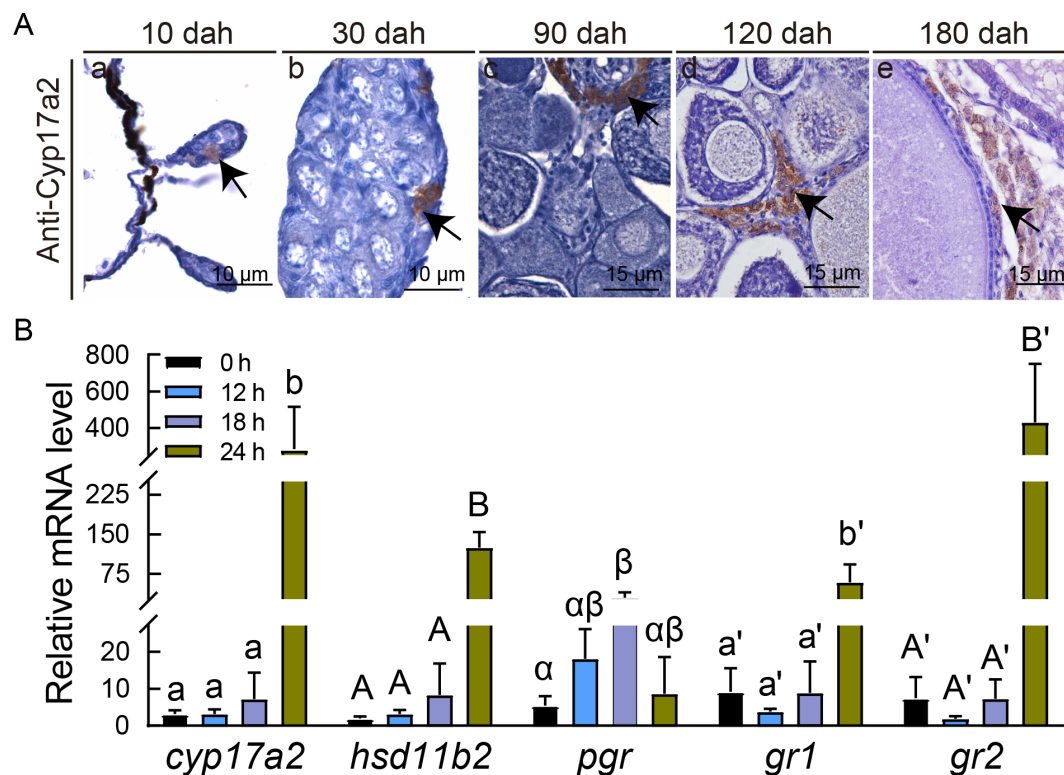


Figure 1 Expression profile of Cyp17a2 in tilapia ovary and effects of human chorionic gonadotropin (hCG) induction on expression of 17,20 β -dihydroxy-4-pregnen-3-one (DHP) and cortisol synthases and receptors

A: Expression of Cyp17a2 in tilapia ovaries at different developmental stages, as determined by immunohistochemistry. Arrow indicates positive signals of Cyp17a2. Dah, days after hatching. B: Expression levels of *cyp17a2*, *pgr*, *hsd11b2*, *gr1*, and *gr2* in ovary of wild-type XX individuals at different time points during hCG treatment *in vitro*. β -actin was used as a reference gene to normalize expression values. Data are reported as mean $\pm$ SD. Different letters above error bars for each gene indicate statistical differences determined by one-way ANOVA followed by Tukey's *post-hoc* test ($P < 0.05$).

and ovulation were explored using *in vitro* and *in vivo* experiments. The *in vitro* experiments demonstrated that fully grown immature oocytes from *cyp17a2*^{+/+} XX females exhibited GVBD in response to hCG treatment, indicating successful entry into the maturation stage (Figure 4A, B; Supplementary Figure S3). In contrast, GVBD did not occur in ovarian fragments from *cyp17a2*^{-/-} XX females when exposed to hCG, indicating a failure in oocyte maturation due to *cyp17a2* gene deficiency (Figure 4A, B). However, supplementation with DHP in conjunction with hCG induced oocyte maturation in *cyp17a2*^{-/-} XX females (Figure 4A, B).

The findings were verified by *in vivo* experiments, where mature oocytes undergoing GVBD were successfully obtained from *cyp17a2*^{-/-} XX females following 4-month co-treatment with hCG, cortisol, and DHP. Mature oocytes were extracted by gentle abdominal pressure, and they remained enclosed within the follicular cell layer, exhibiting strong immunoreactivity for Cyp19a1a (Figure 4C, D). However, no eggs were obtained from *cyp17a2*^{-/-} XX fish treated with 17 α -OHP+hCG or DHP+hCG via abdominal squeezing (data not shown).

To further examine the impact of *cyp17a2* deficiency, histological analysis (Figure 5A) and schematic representation (Figure 5B) of follicular cells were performed for pre-ovulatory wild-type (CTRL), post-ovulatory wild-type (CTRL-S), *cyp17a2*^{-/-}, and steroid-rescued *cyp17a2*^{-/-} XX females (*cyp17a2*^{-/-}-R) prior to gene expression assay. The RT-qPCR results showed that, compared to the control, the expression levels of *mmp9*, *adamts13*, and *collagenase3* were

significantly up-regulated and the expression levels of *col1a1* and *col1a1b* were significantly down-regulated in follicular cells at the final maturation and ovulation stages of regular post-ovulatory fish (CTRL-S; Figure 5C). Notably, *cyp17a2* gene deficiency disrupted the expression pattern of these genes, regardless of steroid rescue treatment (Figure 5C).

Effects of *cyp17a2* gene mutation on steroidogenesis-related gene expression in follicular cell layers

The RT-qPCR results revealed a significant decrease in the expression of key steroidogenic enzymes, including *cyp11a1*, *cyp19a1a*, *StAR2*, and *cyp17a1*, in the follicular cells of post-ovulatory XX fish (CTRL-S) compared to pre-ovulatory fish (CTRL; Figure 6A). When contrasted with preovulatory wild-type female fish (CTRL), *cyp17a2*^{-/-} and *cyp17a2*^{-/-}-R XX females showed no significant differences in the expression of *cyp11a1*, *cyp19a1a*, *cyp19a1b*, and *cyp17a1* in follicular cells, although abnormal up-regulation of *StAR1* and *StAR2* was observed in *cyp17a2*^{-/-}-R and *cyp17a2*^{-/-} XX fish, respectively (Figure 6A). Additionally, *cyp17a2*^{-/-} females exhibited up-regulation in the expression of gonadotropin receptors (*fshr* and *lhr*), steroidogenic factor-1 (*sf1*), and nuclear progesterone receptor (*pgr*) compared to preovulatory (CTRL) and post-ovulatory wild-type females (CTRL-S) (Figure 6B). Notably, DHP and cortisol supplementation normalized the expression levels of *fshr* and *lhr* in *cyp17a2*^{-/-}-R XX fish (Figure 6B).

Steroid hormone metabolism analysis further indicated that E2, cortisone, cortisol, and androstenedione levels were significantly lower in wild-type females at the oocyte

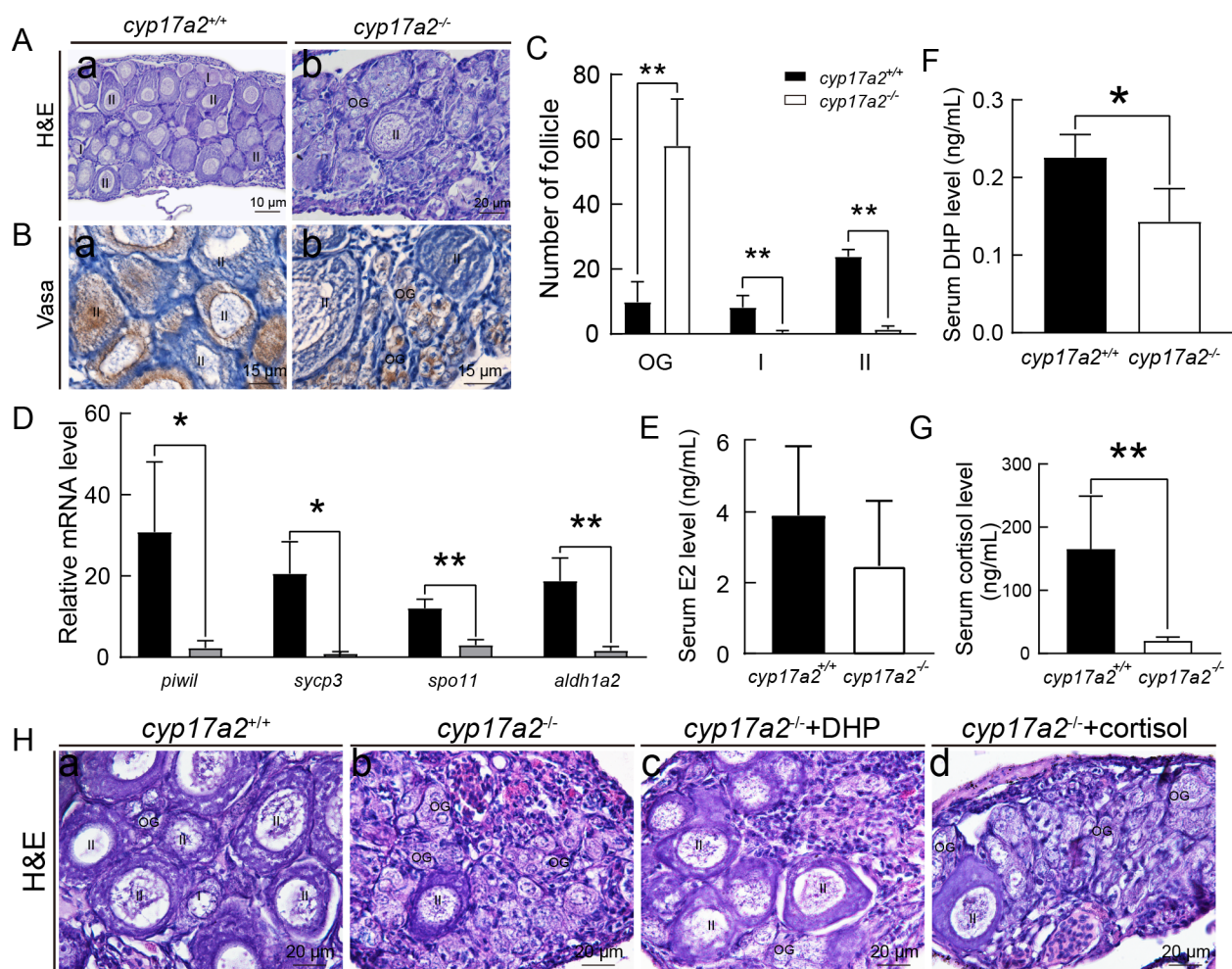


Figure 2 Mutation of *cyp17a2* delayed oogenesis at 90 days after hatching (dah)

A: Histological analysis of *cyp17a2*^{+/+} (a) and *cyp17a2*^{-/-} (b) XX fish ovaries using hematoxylin-eosin (H&E) staining. B: Vasa expression in *cyp17a2*^{+/+} (a) and *cyp17a2*^{-/-} (b) XX fish (*n*=3 fish/genotype), as determined by immunohistochemistry. C: Comparison of germ cells at different stages in *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish. D: Expression level of *piwil*, *sycp3*, *spo11*, and *aldh1a2* in ovaries of *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish, as determined by RT-qPCR. E–G: Serum 17 β -estradiol (E2), 17,20 β -dihydroxy-4-pregnen-3-one (DHP), and cortisol levels in *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish (*n*=4 fish/genotype), as determined using an enzyme-linked immunosorbent assay (ELISA). Data are reported as mean $\pm$ SD. Differences between groups were determined using unpaired two-tailed Student's *t*-tests. *: *P*<0.05; **: *P*<0.01. H: Histological analysis of *cyp17a2*^{+/+} (a), *cyp17a2*^{-/-} (b), DHP-treated *cyp17a2*^{-/-} (c), and cortisol-treated *cyp17a2*^{-/-} (d) XX fish ovaries by H&E staining. OG, oogonia; I and II, phase I and II oocytes.

maturation stage (CTRL-M) compared to those at the follicle growth stage (CTRL-G; Figure 7). Compared to CTRL-G, serum 17 α -hydroxyprogesterone levels were significantly reduced and 17 α -hydroxypregnenolone, cortisone, and cortisol were undetectable in *cyp17a2*^{-/-} XX fish (Figure 7).

***Cyp17a2* mutation impairs oocyte maturation via PI3K-Akt signaling pathway dysregulation**

To elucidate the mechanisms by which *cyp17a2* mutation impairs oocyte maturation and ovulation, the effects of *cyp17a2* deficiency on the PI3K-Akt signaling pathway and the role of this pathway on oocyte maturation were analyzed. Results indicated that *cyp17a2* mutation in female tilapia significantly reduced Akt phosphorylation in the ovaries, with expression levels partially restored in steroid-rescued *cyp17a2*^{-/-} females (Figure 8A). Additionally, in wild-type XX fish, hCG-induced *in vitro* oocyte maturation was associated with increased Akt phosphorylation (Figure 8B, C). However, in *cyp17a2*^{-/-} fish, hCG treatment did not stimulate the PI3K-Akt signaling pathway, as shown by RT-qPCR and western

blot analyses (Figure 8B, C).

Both wild-type and *cyp17a2*^{-/-} female ovaries were subsequently treated with a PI3K-Akt signaling pathway inhibitor (LY294002) or agonist (740 Y-P). As expected, LY294002 suppressed Akt phosphorylation in wild-type ovaries (Figure 8D, E), leading to a decrease in the hCG-induced oocyte maturation rates *in vitro* (Figure 8F, G). Conversely, treatment with the agonist 740 Y-P successfully rescued the oocyte maturation defects associated with *cyp17a2* deficiency (Figure 8H, I).

DISCUSSION

Steroid hormones, including progestins and glucocorticoids, are necessary for oocyte maturation and ovulation in teleosts. *Cyp17a2* plays an essential role in the synthesis of C21 steroids in fish, as evidenced by our previous gene editing model conducted on male tilapia (Yang et al., 2022). Notably, a sharp elevation in *cyp17a2* expression in granulosa cells during the critical period of oocyte maturation suggests its

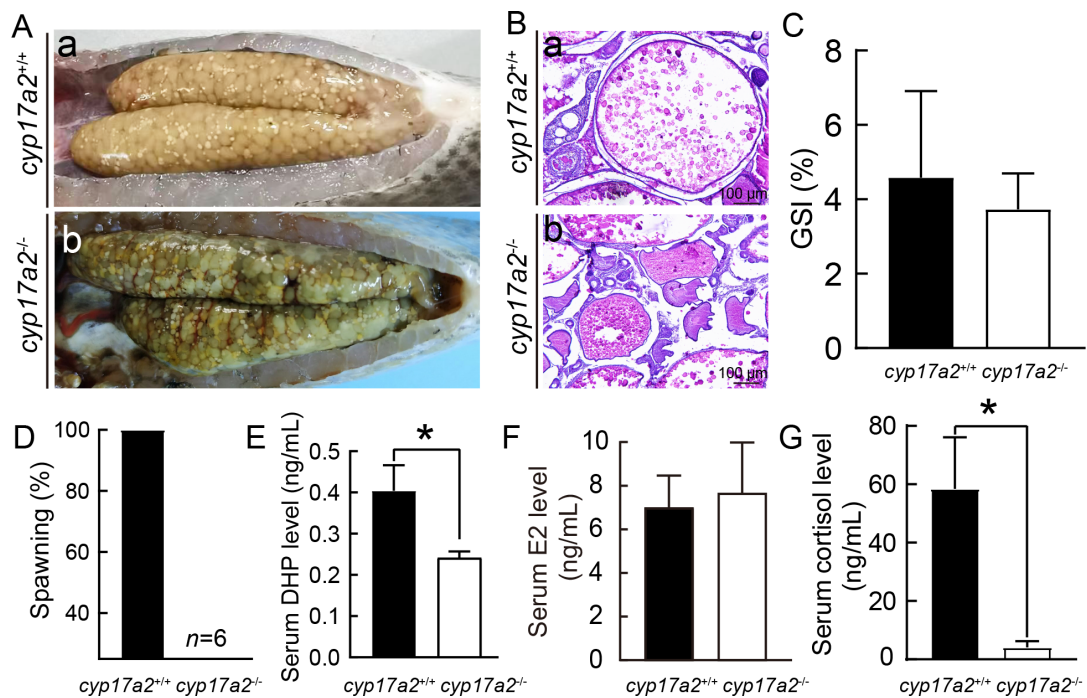


Figure 3 *Cyp17a2* mutation resulted in female spawning failure

A: Morphological observations of *cyp17a2^{+/+}* (a) and *cyp17a2^{-/-}* (b) XX fish. B: Histological analysis of *cyp17a2^{+/+}* (a) and *cyp17a2^{-/-}* (b) XX fish ovaries using hematoxylin-eosin (H&E) staining. C: Comparison of gonadosomatic index (GSI) between *cyp17a2^{+/+}* and *cyp17a2^{-/-}* XX fish at 360 days after hatching (dah). D: Data regarding spawning behavior of *cyp17a2^{+/+}* and *cyp17a2^{-/-}* XX fish ($n=6$ fish/genotype). E–G: Serum 17,20 β -dihydroxy-4-pregnen-3-one (DHP), 17 β -estradiol (E2), and cortisol levels in *cyp17a2^{+/+}* and *cyp17a2^{-/-}* XX fish ($n=5$ fish/genotype), as determined by enzyme-linked immunosorbent assay (ELISA). Data are reported as mean $\pm$ SD. Differences between groups were determined using unpaired two-tailed Student's *t*-tests. *: $P<0.05$.

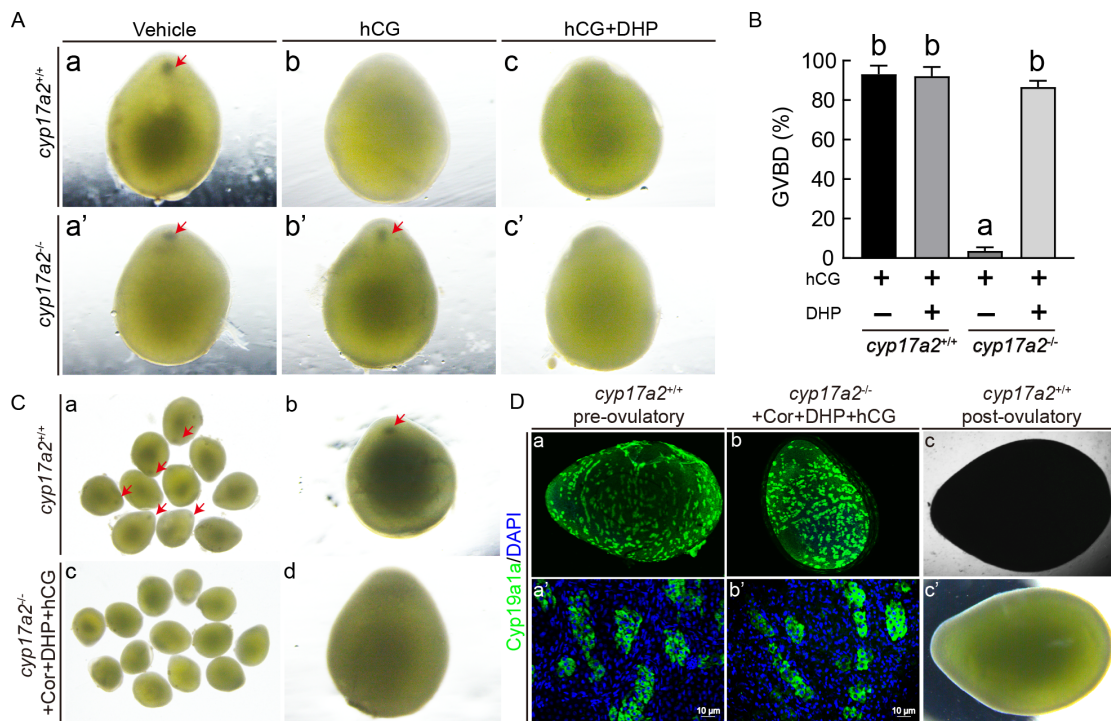


Figure 4 Steroid-rescue of oocyte maturation and spawning in *cyp17a2^{-/-}* fish

A: Oocyte status of *cyp17a2^{+/+}* (a–c) and *cyp17a2^{-/-}* (a'–c') XX fish following *in vitro* treatment with human chorionic gonadotropin (hCG) and 17,20 β -dihydroxy-4-pregnen-3-one (DHP). B: Analysis of oocyte maturation ratio. Data are reported as mean $\pm$ SD. Different letters above error bars for each gene indicate statistical differences determined by one-way ANOVA followed by Tukey's *post-hoc* test ($P<0.05$). C: Oocyte status of *cyp17a2^{+/+}* (a, b) and steroid-rescued *cyp17a2^{-/-}* (c, d) XX fish. D: Expression levels of Cyp19a1a in preovulatory *cyp17a2^{+/+}* XX (a, a'), steroid-rescued *cyp17a2^{-/-}* XX (b, b'), and post-ovulatory *cyp17a2^{+/+}* XX fish (c, c') determined via immunofluorescence (IF) staining. Red arrow indicates germinal vesicle of oocyte. Green and blue fluorescence indicate Cyp19a1a and DAPI signals, respectively.

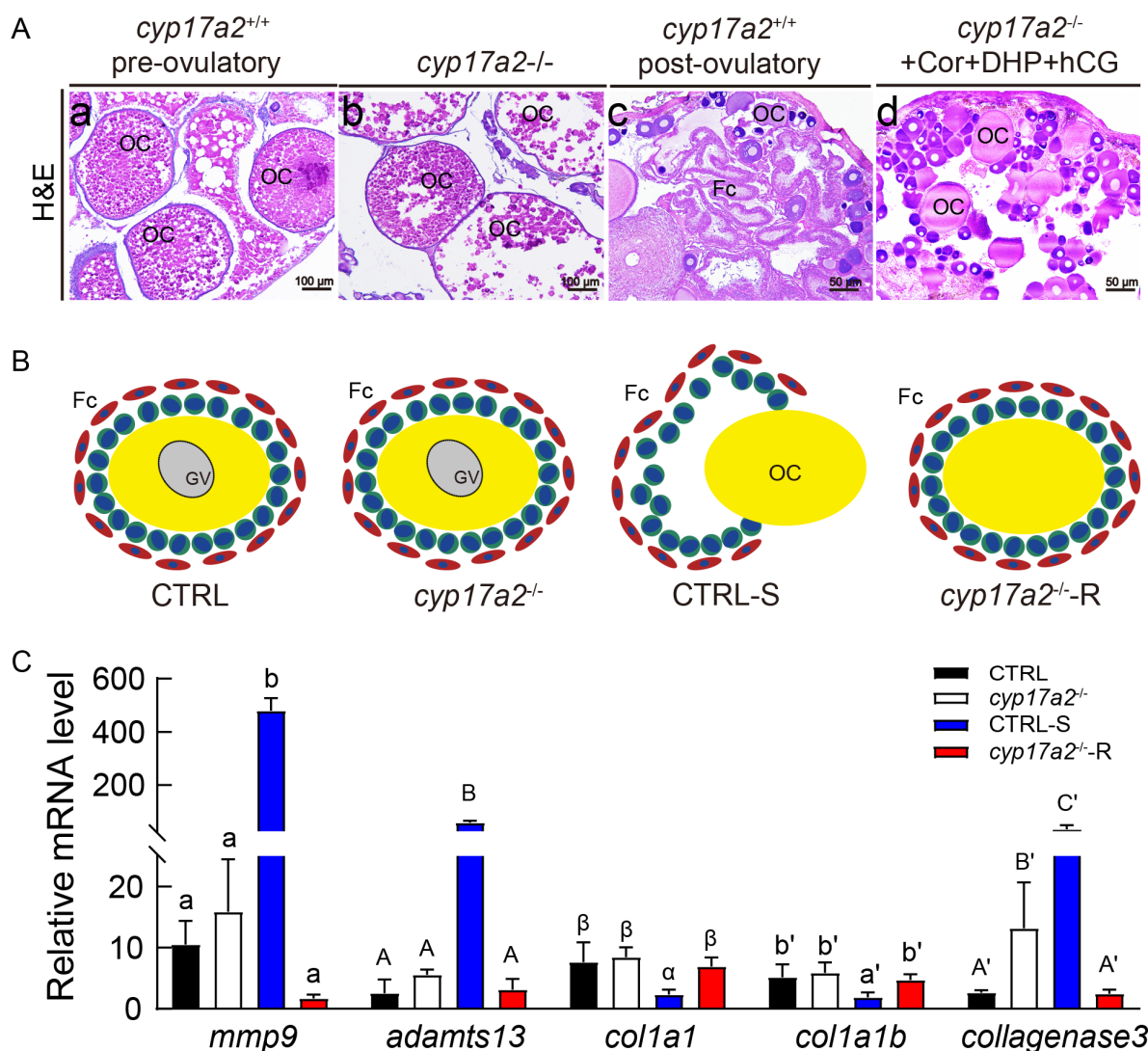


Figure 5 Histomorphology of ovary after spawning and gene expression profiles of cytoplasmic matrix and related enzymes in follicular cells

A: Hematoxylin-eosin (H&E) staining of pre-ovulatory *cyp17a2*^{+/+} (a), *cyp17a2*^{-/-} (b), post-ovulatory *cyp17a2*^{+/+} (c), and steroid-rescued *cyp17a2*^{-/-} (d) XX fish ovaries. B: Schematic of follicular cell sampling. GV, germinal vesicle; Fc, follicular cells. C: Expression levels of *mmp9*, *adamts13*, *col1a1*, *col1a1b*, and *collagenase3* in follicular cells of *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish, as determined by RT-qPCR. β -actin was used as a reference gene to normalize expression values. Data are reported as mean $\pm$ SD. Different letters above error bars for each gene indicate statistical differences determined using one-way ANOVA followed by Tukey's *post-hoc* test ($P < 0.05$). CTRL, follicular cells of pre-ovulatory wild-type females; *cyp17a2*^{-/-}, follicular cells of *cyp17a2*^{-/-} females; CTRL-S, follicular cells of post-ovulatory wild-type females; *cyp17a2*^{-/-}-R, follicular cells of steroid-rescued *cyp17a2*^{-/-} females.

potential involvement in fish oocyte maturation and ovulation (Zhou et al., 2007a). However, the specific functions and mechanisms of *cyp17a2* in female reproduction, particularly in oocyte maturation and ovulation, remain unclear. In the present study, we demonstrated that *cyp17a2* deficiency disrupted DHP and cortisol production, leading to impaired fish oogenesis, oocyte maturation, and ovulation. Supplementation with DHP or DHP/cortisol successfully restored oocyte maturation in *cyp17a2*^{-/-} females, although ovulation was not restored. These findings indicate the involvement of Cyp17a2 in the synthesis of DHP and cortisol, both crucial for fish oocyte maturation and ovulation.

In vertebrates, meiotic initiation is essential for ovarian development and fertility, triggering mitotic-to-meiotic transition in XX individuals. Steroids and other substances like retinoic acid (RA), E2, cortisol, and DHP, are implicated in the regulation of meiotic initiation, oogenesis, and oocyte quality in

fish (Amer et al., 2001; Lubzens et al., 2010; Miura et al., 2007; Nagahama et al., 1995; Xiao et al., 2022; Yan et al., 2019). DHP, in particular, has been identified as a vital signaling steroid for meiotic initiation, promoting the synthesis of DNA, formation of synaptonemal complexes, and expression of meiosis-specific markers (Miura et al., 2006, 2007). Recent research has also revealed the importance of cortisol in oogenesis and female fertility. For example, mutation of the *cyp11c1* gene, encoding a key steroidogenic enzyme involved in glucocorticoid synthesis, has been shown to lead to a decline in glucocorticoids and estrogen levels, inhibiting phase II to phase III transition in female fish (Xiao et al., 2022). Consistent with these findings, our results showed that *cyp17a2* mutation in tilapia impaired DHP and cortisol production without affecting E2 synthesis during early development. Additionally, oogenesis was arrested in *cyp17a2*^{-/-} XX fish at 90 dah, with only limited oocytes

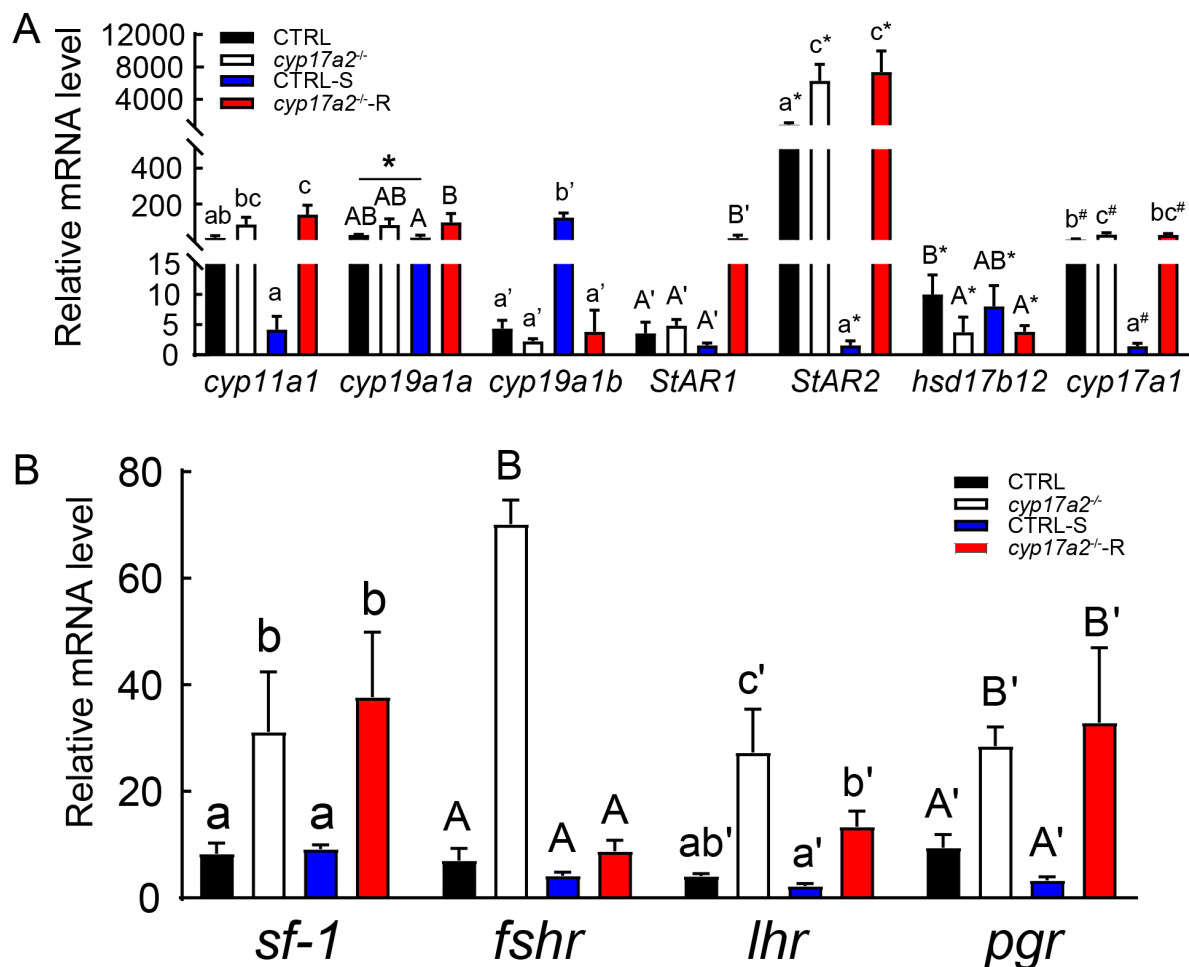


Figure 6 Mutation of *cyp17a2* disrupted steroidogenesis in female tilapia

Expression levels of steroidogenic enzymes (*cyp11a1*, *cyp19a1a*, *cyp19a1b*, *StAR1*, *StAR2*, *hsd17b12*, and *cyp17a1*) (A) and transcription factor gonadotropin receptors (*sf-1*, *fshr*, *lhr*, and *pgr*) (B) in follicular cells of *cyp17a2*^{+/+} and *cyp17a2*^{-/-} XX fish ($n=3$ fish/group), as determined by RT-qPCR. β -actin was used as a reference gene to normalize expression values. Data are reported as mean $\pm$ SD. Differences between two groups were determined by unpaired two-tailed Student's *t*-tests (*: $P<0.05$). Different letters above error bars for each gene indicate statistical differences determined using one-way ANOVA followed by Tukey's *post-hoc* test ($P<0.05$). CTRL, follicular cells of pre-ovulatory wild-type females; *cyp17a2*^{-/-}, follicular cells of *cyp17a2*^{-/-} females; CTRL-S, follicular cells of post-ovulatory wild-type females; *cyp17a2*^{-/-}-R, follicular cells of steroid-rescued *cyp17a2*^{-/-} females.

progressing to phase II and a significant decline in the expression of germ cell marker (*piwil*) and meiosis-related genes (*spo11*, *sycp3*, and *aldh1a2*). Rescue experiments confirmed that DHP, but not cortisol, partially alleviated the delay in meiotic initiation observed in *cyp17a2*^{-/-} female fish. These findings further emphasize the significance of DHP in driving meiotic initiation in fish.

Sex determination in vertebrates is a highly complex process, broadly categorized into environmental sex determination, genetical sex determination, or a combination of both (Li et al., 2019; Zhu et al., 2022). In some species, meiosis initiation or germ cell number are associated with sex differentiation (Adolfi et al., 2021; Aharon and Marlow, 2022). In tilapia, however, neither the inhibition of RA synthesis or metabolism, nor the suppression of germ cell proliferation via gene knockout or inhibitor treatment, have been shown to alter the sex ratio (Feng et al., 2015; Li et al., 2023; Xiao et al., 2022). In this study, mutation of *cyp17a2* led to delayed meiosis in tilapia without impacting sex differentiation, and the arrested oogenesis was restored by 180 dah, indicating that normal E2 levels may be sufficient to sustain oogenesis. Additionally, our results revealed that the inhibition of both

DHP and cortisol did not impact E2 production, supporting the independence of C18 and C21 steroidogenic pathways in fish. Importantly, both C18 and C21 steroids appear indispensable for oogenesis, acting synergistically to promote ovarian development.

Accumulating evidence indicates that DHP triggers final oocyte maturation and ovulation via membrane progesterone receptors (mPRs) nongenomic and nuclear progesterone receptor genomic pathways (Nagahama & Yamashita, 2008; Zhu et al., 2008). In zebrafish, homozygous *pgr*^{-/-} female fish are infertile and fail to ovulate, even following hCG or DHP treatment *in vitro* (Tang et al., 2016). However, oocytes from *pgr*-mutant females treated with hCG or DHP successfully undergo GVBD and final maturation both *in vivo* and *in vitro* (Zhu et al., 2015). Additionally, impaired oocyte maturation and reduced GVBD ratios occur in *mprs*-mutant zebrafish (Wu et al., 2020). In tilapia, the temporally regulated expression of *cyp17a1* and *cyp17a2* during the steroidogenic transition from E2 to DHP during the spawning cycle suggests that Cyp17a2 is a key steroidogenic enzyme for DHP production (Zhou et al., 2007a). In this study, *cyp17a2* deficiency led to a significant decline in serum DHP levels in mature females, resulting in

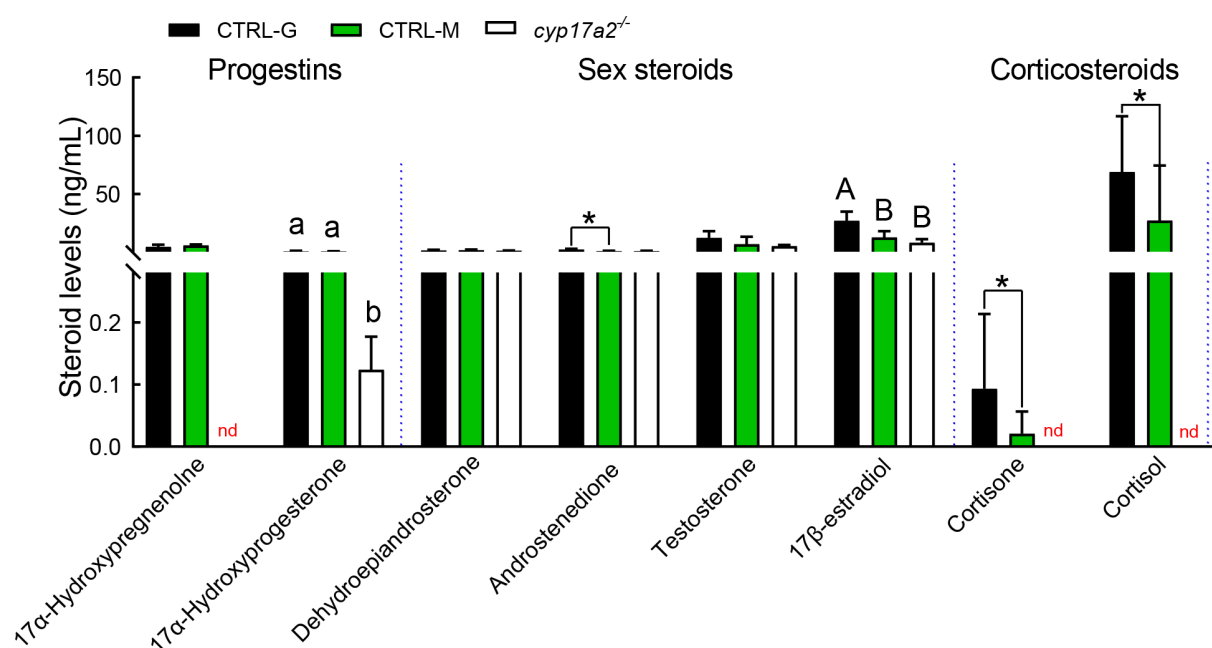


Figure 7 Effects of *cyp17a2* deficiency on steroid production in adult female tilapia (approximately one year old)

Serum steroid levels in wild-type females at follicular growth stage ($n=3$) and oocyte maturation stage ($n=3$) and in *cyp17a2*^{-/-} females ($n=3$), as determined by LC-MS. Data are reported as mean $\pm$ SD. Differences between two groups were determined by unpaired two-tailed Student's *t*-tests (*: $P<0.05$). Different letters above error bars for each hormone indicate statistical differences determined using one-way ANOVA followed by Tukey's *post-hoc* test ($P<0.05$). CTRL-G, wild-type females at follicle growth stage; CTRL-M, wild-type females at oocyte maturation stage; nd, not detected.

GVBD and ovulation failure. However, supplementation with DHP successfully rescued GVBD and oocyte maturation, indicating that DHP likely plays an indispensable role in fish oocyte maturation. Reproductive strategies and spawning habits play key roles in fish evolution, with various species exhibiting different spawning behaviors (Cao et al., 2023). Many cichlid species, such as tilapia, exhibit typical nest-building and mouthbrooding behaviors. *Cyp17a2* deficiency has been linked to the cessation of nesting behavior, implicating a critical role for DHP in the reproductive cycle of fish. Although exogenous DHP or combined DHP and hCG administration restored oocyte maturation in *cyp17a2*^{-/-} females, it did not fully restore ovulation or nesting behavior. These findings suggest that fish ovulation is a complex process likely governed by multiple signaling pathways and interacting factors.

Cortisol, the primary glucocorticoid in fish, is increasingly recognized as a significant regulator of oocyte maturation and ovulation (Faught & Vijayan, 2018). For instance, female zebrafish lacking *cyp11a1* show reduced egg spawning and impaired *in vitro* GVBD, both partially rescueable by cortisol supplementation (Zhang et al., 2020). Additionally, GR deficiency in zebrafish accelerates ovarian aging, characterized by follicular atresia in vitellogenin oocytes, yolk liquefaction, and large inflammatory infiltrates (Faught et al., 2020). In *cyp11c1*^{-/-} female tilapia, cortisol deficiency results in disrupted vitellogenesis, arrested oogenesis, and increased follicular cell apoptosis (Xiao et al., 2022). Our results revealed a significant up-regulation in the steroidogenic enzyme *hsd11b2* and the *gr* gene within 24 h of hCG induction, indicating the potential involvement of glucocorticoid-GR signaling in fish oocyte maturation and ovulation. The observed sharp decline in serum cortisol levels highlights the essential role of *Cyp17a2* in cortisol production, which may subsequently impact ovulation. Notably,

supplementation with hCG, DHP, and cortisol successfully stimulated the release of mature oocytes upon abdominal pressure. However, despite this stimulation, *cyp17a2*^{-/-} oocytes did not successfully ovulate from the surrounding follicular cell layers, as evidenced by the strong positive signals of *cyp19a1a* gene expression.

Previous research has demonstrated that ovulation is a dynamic tissue remodeling process, wherein mature oocytes are ultimately released from the cleavage of extracellular matrix (ECM) and the rupture of basal membranes and follicular layers. Metalloproteinases (such as MMPs and Adamts) are essential regulators of follicular rupture ovulation in teleosts (Duffy et al., 2019). In zebrafish, the transcripts of metalloproteinases, particularly *adamts9* and *mmp9*, are significantly elevated in follicular cells of preovulatory wild-type females, whereas homozygous *pgr* mutants exhibit reduced metalloproteinase expression, resulting in anovulation (Liu et al., 2018). In our recent study, we found high expression of ECM components (*cola1a* and *col1a1b*) and a significant decline in proteinases (*mmp9* and *adamts13*) and *collagenase3* in the ovaries of *cyp17a2*^{-/-} XX females, potentially accounting for the anovulation observed in these mutants. Furthermore, excessive DHP and cortisol supplementation failed to rescue ovulation, indicating the complexity of fish ovulation. We propose that the anovulation observed in *cyp17a2*^{-/-} females, likely exacerbated by the reduction in DHP and cortisol, may also involve other signaling pathways that cannot be reversed by steroids. Future studies will incorporate additional relevant mutants and employ multi-omics approaches to unravel the molecular regulatory networks underlying oocyte maturation and ovulation in fish.

During the teleost spawning cycle, distinct patterns in steroid hormone conversion and steroidogenic enzyme expression are evident (Crews, 1984; Nagahama et al., 1993). Zebrafish studies have shown significant up-regulation of

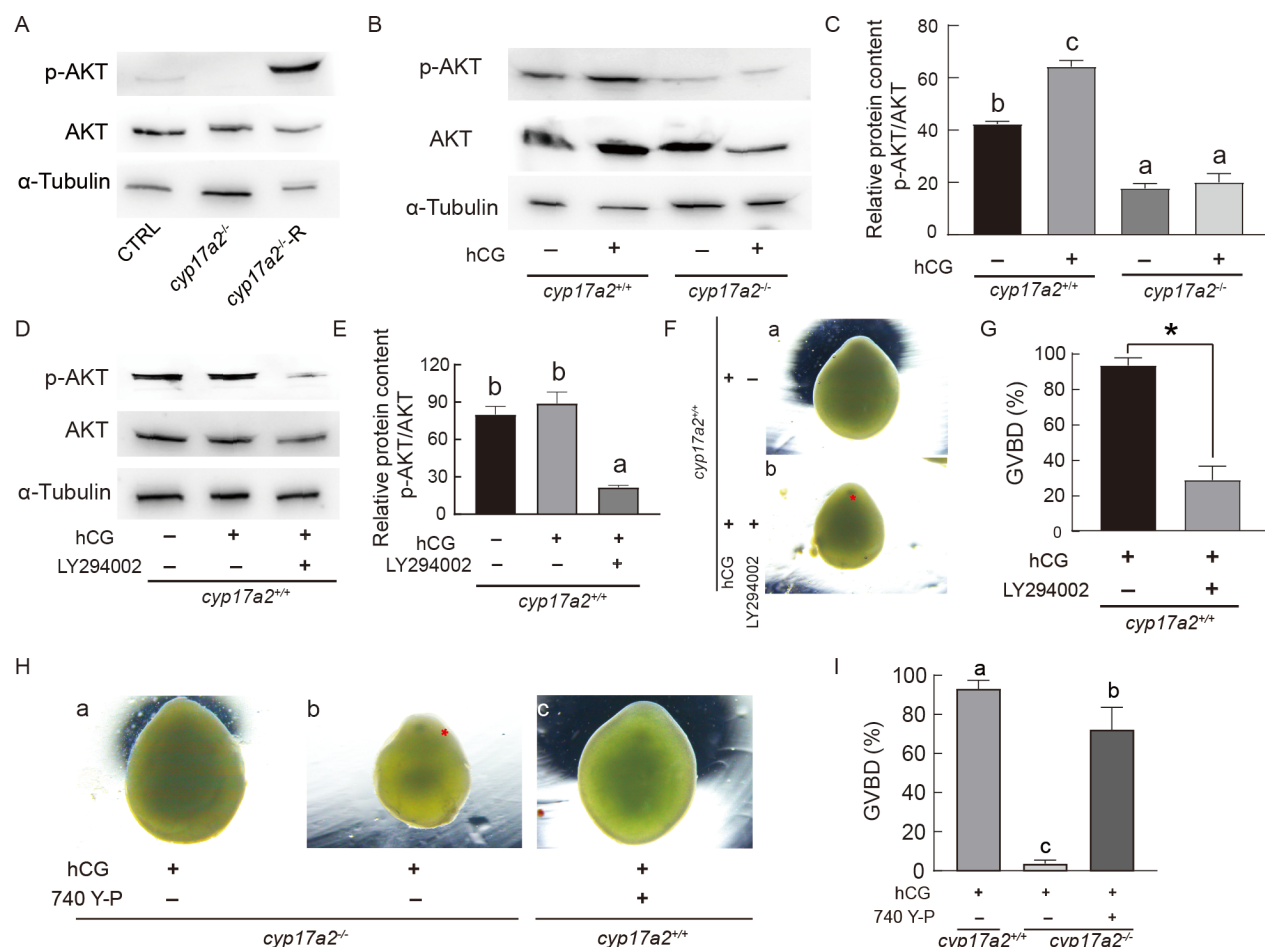


Figure 8 *Cyp17a2* deficiency inhibited protein kinase B (Akt) phosphorylation, essential for oocyte maturation in female tilapia

A: Expression of Akt and phospho-Akt (p-Akt) in ovaries of *cyp17a2*^{+/+}, *cyp17a2*^{-/-}, and steroid-rescued *cyp17a2*^{-/-} female tilapia, as determined by western blotting. B, C: Expression and densitometry analysis of Akt and p-Akt in ovaries of *cyp17a2*^{+/+} and *cyp17a2*^{-/-} female tilapia, as determined by western blotting. D, E: Expression and densitometry analysis of Akt and p-Akt in ovaries of *cyp17a2*^{+/+} XX fish treated with LY294002 *in vitro*, as determined by western blotting. F: Observation of oocyte status under LY294002 treatment *in vitro*. G: Statistical analysis of oocyte maturation ratio under LY294002 treatment. H: Observation of oocyte status under 740 Y-P treatment *in vitro*. I: Statistical analysis of oocyte maturation ratio under 740 Y-P treatment. Data are reported as mean±SD. Differences between two groups were determined by unpaired two-tailed Student's *t*-tests (*: *P*<0.05). Different letters above error bars indicate statistical differences determined by one-way ANOVA followed by Tukey's *post-hoc* test (*P*<0.05).

steroidogenic enzymes, such as *hsd17b1*, *hsd17b3*, and *cyp19a1a*, during early vitellogenesis, and significant down-regulation in the expression of *StAR*, *cyp11a1*, *hsd3b1*, and *cyp17a1* with maturation (Ings & Van Der Kraak, 2006). In contrast, *cyp17a2* and *hsd17b12* expression and DHP production are significantly elevated during the oocyte maturation stage in fish, suggesting the involvement of Cyp17a2 in DHP production (Aranyakanont et al., 2020; Zhou et al., 2007a). In this study, *cyp17a2* mutation disrupted this steroidogenic shift in female tilapia, as evidenced by the high *cyp19a1a* expression in follicular cells and E2 production throughout the 14 day reproductive cycle. Using a *cyp17a2*^{-/-} mutation line, we demonstrated that the increase in DHP production during the oocyte maturation and ovulation stages may be required for the suppression of E2 production.

The PI3K/Akt signaling pathway plays a crucial role in cell cycle regulation, particularly in ovarian development, with Akt expression in both oocytes and somatic cells supporting primordial germ cell proliferation and meiotic progression (Kalous et al., 2009; Kimura et al., 2008). During oocyte meiotic maturation, both Akt and the maturation-promoting

factor were increased (Alcaraz et al., 2022; Kalous et al., 2023). Previous *in vivo* and *in vitro* analyses have confirmed that Akt phosphorylation is necessary to resume meiosis (Han et al., 2006; Newhall et al., 2006; Prochazka et al., 2012). In female mice, *Akt1* deficiency impairs fertility, disrupting follicular development and oocyte growth (Brown et al., 2010). Moreover, incubation of neonatal mouse ovaries with the PI3K-activating peptide 740Y-P enhances Akt phosphorylation and stimulates follicle activation (Hao et al., 2024; Li et al., 2010). In this study, *cyp17a2* mutation in tilapia reduced Akt phosphorylation in the ovaries. Additionally, *in vitro* experiments demonstrated that the PI3K-Akt signaling pathway inhibitor LY294002 decreased oocyte maturation rates in wild-type females, while a PI3K-Akt signaling pathway agonist restored oocyte maturation defects in *cyp17a2*-deficient females. These results suggest that the PI3K-Akt signaling pathway may act downstream of DHP, which is synthesized by Cyp17a2, to regulate oocyte maturation and ovulation in fish.

Taken together, our findings substantiate the crucial involvement of Cyp17a2 in DHP biosynthesis in the ovary and

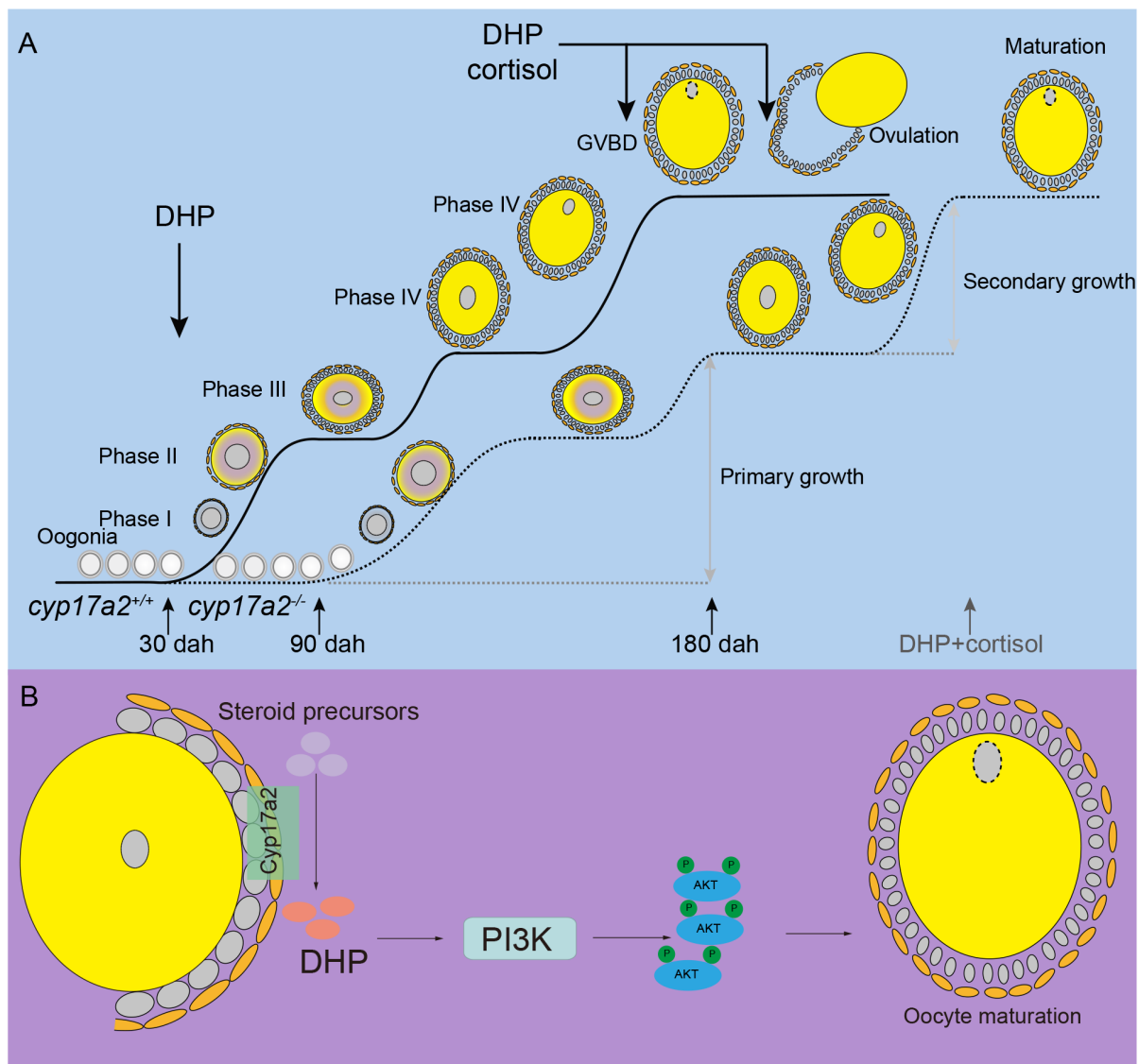


Figure 9 Potential mechanisms of Cyp17a2 in fish oogenesis, oocyte maturation, and ovulation

A: Homozygous mutation of *cyp17a2* in female tilapia arrested oogenesis due to a significant decrease in serum 17,20β-dihydroxy-4-pregnen-3-one (DHP). With fish development, ovarian development in *cyp17a2*^{-/-} fish showed signs of recovery at 180 days after hatching (dah). However, the absence of DHP and cortisol in *cyp17a2*^{-/-} XX fish impaired oocyte maturation and ovulation. Administration of exogenous DHP and cortisol successfully restored oocyte maturation, but mature oocytes remained entrapped within surrounding follicular cells. B: Cyp17a2 expressed in follicular cells catalyzes steroid precursors into DHP, which plays a crucial role in regulating oocyte maturation through the PI3K-Akt signaling pathway. GVBD, germinal vesicle breakdown; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; P, phosphorylation.

cortisol production in the head kidney of female tilapia, as well as the promotion of oocyte maturation and ovulation by activating Akt phosphorylation (Figure 9).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

L.Y.Y. and L.Y.Z. conceived and designed the experiments. L.Y.Y. performed most of the experiments. Y.W. and X.F.Z. conducted the rescue experiments. S.H.S. established the chimeric mutant of the *cyp17a2* gene. L.Y.Y. wrote the initial manuscript. L.Y.Z., J.X., and D.S.W. revised and approved the article. All authors read and approved the final version of the manuscript.

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