



Dual *cyp17a1/a2* knockout unveils paralog-specific steroid pathways in fish reproduction

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ABSTRACT

Cyp17a is a key enzyme in steroidogenesis and reproduction in vertebrates. In teleosts, two paralogous genes, *cyp17a1* and *cyp17a2*, have been identified, and while the potential impact of mutations in these genes have been studied, a comprehensive comparative analysis of their regulatory mechanisms in teleost reproduction remains lacking. In this study, we established a double mutant (dco) of *cyp17a1* and *cyp17a2* and conducted a transcriptome analysis of their gonads to systematically investigate the differences in steroidogenesis and gene expression between the two genes. Phenotypic analysis revealed that both *cyp17a1*^{-/-} and dco mutants exhibited a reduced gonadosomatic index, abnormal Leydig cell proliferation, and male infertility. RNA sequencing of the testes identified 2688 differentially expressed genes (DEGs), with significant variations between the mutants and wild-type males. KEGG enrichment analysis indicated that DEGs were enriched in pathways related to steroid biosynthesis, HIF-1, PPAR, and Hippo signaling. Further, steroid profiling confirmed *cyp17a1*^{-/-}-specific androgen and estrogen deficiency (T, 11-KT, E1, and E2), while revealing dco-specific deficiencies in DHP, cortisol, and cortisone, alongside an overaccumulation of corticosterone, highlighting paralog-specific substrate preferences. These findings underscore the Cyp17a paralogs as critical regulators of testicular metabolic-transport networks, thereby offering insights into male infertility linked to steroid biosynthesis defects.

1. Introduction

Steroid hormones, such as estrogens, androgens, glucocorticoids, and progestins, are crucial endocrine regulators that play vital roles in gonadal development, gametogenesis, and fertility in vertebrates, with their biosynthesis being largely conserved between mammals and fish [1–4]. The synthesis of steroid hormones from cholesterol is facilitated by a highly conserved cascade of cytochrome P450 enzymes (CYPs) and hydroxysteroid dehydrogenases (HSD). Among these, Cytochrome P450 17- α -hydroxylase/C(17,20)-lyase (P450c17/Cyp17) serves as a crucial member of the cytochrome P450 subfamily, possessing both 17- α -hydroxylase and 17,20 lyase activities, and plays essential roles in the production of C-18, -19, and -21 steroids [5]. The 17- α -hydroxylase activity catalyzes the conversion of pregnenolone to 17- α -hydroxypregnenolone or/and progesterone to 17- α -hydroxyprogesterone. Subsequently, its 17,20-lyase activity facilitates the cleavage of the C17,20 bond transforming 17- α -hydroxypregnenolone and 17-

α -hydroxyprogesterone into dehydroepiandrosterone (DHEA) and androstenedione, respectively [6–8].

In mammals, numerous studies have demonstrated that Cyp17a plays a vital role in the biosynthesis of glucocorticoid in the adrenal glands and the production of sex steroid in the gonads, as evidenced by inhibitor treatments and gene mutations [9–11]. In teleosts, the unique third-round genome duplication event in bony fish has resulted in two duplicated *cyp17a* genes, named *cyp17a1* and *cyp17a2*. These two genes display distinct expression patterns in tissues, with their encoded proteins differing in enzyme activities. Cyp17a1 is primarily expressed in the gonads (follicular cells in the ovaries and Leydig cells in the testes) and exhibits both 17- α -hydroxylase and 17,20-lyase activities, which is essential for the production of C18 (estrogens) and C19 (androgens) steroids. While *cyp17a2* is expressed in both the gonads (follicular cells in the ovaries and Leydig cells in the testes) and the head kidney (interrenal cells), exhibiting only 17- α -hydroxylase activity, which is critical for the synthesis of C21 steroids (DHP and corticosteroids)

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[7,8,12–14].

In Nile tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), and common carp (*Cyprinus carpio*), single-gene mutants of *cyp17a1* and *cyp17a2* have been successfully generated [13–17]. In both tilapia and zebrafish, mutation of the *cyp17a1* gene led to a deficiency in estrogen (17 β -estradiol, E2) and androgens (testosterone, T; 11-ketotestosterone, 11-KT), resulting in female-to-male sex reversal, loss of male-specific secondary sexual characteristics, and impaired mating behaviors, ultimately leading to infertility in male fish, with tilapia showing hypogonadism and zebrafish displaying testicular hypertrophy [14,17]. These findings emphasize the critical role of E2 in female sex differentiation and maintenance, while androgens are essential for the development of secondary sexual characteristics and male fertility. Conversely, mutations in the *cyp17a2* gene had minor effects on male fertility in both species, though alterations in C21 steroid production were noted, suggesting that C21 steroids may not significantly impact male reproductive function [15,18]. These findings highlight that *cyp17a* genes (*cyp17a1* and *cyp17a2*) as critical factors for understanding the influence of steroids on fish reproduction and for advancing aquaculture practices.

In the present study, we generated double mutants of the *cyp17a1* and *cyp17a2* genes by crossbreeding heterozygotes that possessed mutations in either the *cyp17a1* or *cyp17a2* genes. This was undertaken to explore in detail the potential molecular mechanisms through which the *cyp17a1* and *cyp17a2* genes influence reproductive processes, particularly focusing on spermiogenesis and male fertility. To achieve this, we conducted a transcriptome analysis on the testes of three groups: wild-type males, males with a single-gene mutation in *cyp17a1*, and double mutants of *cyp17a1* and *cyp17a2* genes. This analysis helped in identifying and characterizing key pathways and genes crucial for male fertility in tilapia. The pathways and genes uncovered in this analysis offer new insights into the complex regulatory mechanisms that underpin in the reproductive biology of male fish, potentially guiding further research and therapeutic strategies to manage fertility issues in aquatic species.

2. Materials and methods

2.1. Animal and ethics statement

The Nile tilapia, *Oreochromis niloticus*, were adapted to conditions in recirculating aerated freshwater tanks at 26 °C, under natural light cycles before being used in experiments. The fish were fed commercial dry food (Shengsu, Shandong, China) three times daily. All procedures involving animals were carried out following the guidelines stipulated in the Guide for Care and Use of Laboratory Animals, as approved by the Committee of Laboratory Animal Experimentation at Southwest University, China. (Approval No. IACUC-20181015-12, 15 October 2018).

2.2. Generation of double mutant line of *cyp17a1* and *cyp17a2* gene

The homozygous mutations for *cyp17a1* and *cyp17a2* were previously established using the CRISPR/Cas9 system and have been maintained in our laboratory [14,15]. For generation of *cyp17a1*^{-/-}; *cyp17a2*^{-/-} double mutants, the *cyp17a1*^{+/-}; *cyp17a2*^{+/-} double heterozygotes were obtained by crossing *cyp17a1*^{+/-} XX females with *cyp17a2*^{+/-} XY males. Subsequently, the *cyp17a1*^{-/-}; *cyp17a2*^{-/-} double mutants were generated by crossing *cyp17a1*^{+/-}; *cyp17a2*^{+/-} XX females with *cyp17a1*^{+/-}; *cyp17a2*^{+/-} XY males.

2.3. Sampling and histological examination

The experimental fish were anesthetized using tricaine methane sulfonate (MS222, Sangon Biotech, Shanghai, China) and immediately sampled. Prior to histological assessment, the body weight and age of each fish were recorded. After this, the gonads from each fish were

removed, weighed, and the gonadosomatic index (GSI, the ratio of testis weight to body weight) was determined. After collection, the gonads were preserved in Bouin's solution for 12 h at room temperature, then dehydrated and embedded in paraffin wax. The prepared tissues were then cut into 5- μ m-thick sections and stained with hematoxylin and eosin for microscopic examination. The efferent duct area index (the ratio between efferent duct cross-section areas gonad cross-section areas) was calculated using ImageJ software. The following steps were followed: 1) Open ImageJ software. 2) Open the image from file. 3) Set the ruler scale. Click on the “line tool”, draw a line segment along a known distance in the image. 4) Describe the ruler line segment. After drawing the line, click on “Analyze-Set Scale” and the “Distance in pixels” is automatically filled based on the line drawn. 5) Measurement target. Use shapes to outline the contours of the area or object you want to measure. 6) Measure the target. After selecting the area, click on “Analyze-Measure”. The results table will appear showing measurements and be saved for further analysis.

2.4. Immunofluorescence (IF) analysis

The gonads were harvested from wild-type males (control, ctrl), *cyp17a1*^{-/-}; *cyp17a2*^{+/-} males (*cyp17a1*^{-/-}), and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} males (double knockout, dko). IF analysis was carried out using methodologies previously described (Yang et al., 2021). In brief, gonads of these fish were fixed overnight at 4 °C in 4 % paraformaldehyde in PBS with gentle agitation. After fixation, the samples underwent dehydration and were embedded in paraffin. Tissue sections were then cut to a thickness of 5 μ m. These sections were blocked using a solution of 5 % BSA in 1 $\times$ PBS (Sangon Biotech, Shanghai, China), followed by overnight incubation at 4 °C with a primary antibody against StAR (Steroidogenic acute regulatory protein 1, diluted 1:500), a marker for Leydig cells. After primary antibody incubation, sections were washed five times with 1 $\times$ PBS for 5 min each. For negative controls, normal rabbit serum was used in place of the primary antibody. Sections were then treated with an Alexa Fluor 594-conjugated secondary antibody (diluted 1:1000 in blocking solution, Invitrogen, Carlsbad, CA, USA). Nuclei were stained using 40,60-diamidine-2-phenylindole-dihydrochloride (DAPI, Invitrogen). Images were captured with an FV3000 confocal microscope (Olympus, Tokyo, Japan). The percentage of StAR-positive cells was quantified using ImageJ software, based on the total area of stained cells relative to the total area within the images. The following steps were followed: 1) Open ImageJ software; 2) Open the image from file; 3) Click on “Image-Type-RGB Stack” and select the image with the highest matching degree with the positive signal for subsequent analysis. 4) Select “Image-Adjust-Threshold.” The auto setting can be selected; 5) Choose “Dark background” for fluorescence; 6) Click on “Set” to set the threshold of the image; 7) Select “Analyze-Set Measurements” and choose the parameters for fluorescence measurement. Ensure that the “Limit to Threshold” option is selected, otherwise the entire image will be measured, rather than the selected area. 8) Select “Analyze-Measure.” The results table will appear and be saved for further analysis.

2.5. Transcriptome sequencing

The testes from six-month-old wild-type males (ctrl), *cyp17a1*^{-/-}; *cyp17a2*^{+/-} males (*cyp17a1*^{-/-}), and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} males (dko) at 180 dah were collected for transcriptome sequencing, with three samples from each group. For RNA sampled preparation, 1 μ g of RNA per sample was used as the starting material. Sequencing libraries were constructed using the HiSeq NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasten Biotechnology (Shanghai) Co., Ltd.), adhering to the manufacturer's instructions, and index codes were assigned to link sequences back to their respective samples. These libraries were then sequenced on an Illumina NovaSeq 6000 platform to produce 150 bp paired-end reads. The clean reads from each library

were mapped to the reference genome *O. niloticus*_UMD_NMBU (GCA_001858045.3, available at https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_001858045.2/) using the Hisat2 software with standard settings. Gene expression levels were quantified using the Reads Per Kilobase of transcript per Million mapped reads (RPKM) method.

2.6. Identification of differentially expressed genes (DEGs)

Differential expression analysis was conducted using DESeq2. The resulting *p*-value were adjusted for multiple testing using the Benjamini and Hochberg method to control the false discovery rate (FDR). Genes identified by DESeq2 with a fold change of at least 2 and an FDR <0.05 were classified as differentially expressed.

2.7. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment

KEGG pathway analysis was carried out to determine the significantly enriched biological pathways among the DEGs. DEGs were initially selected using a criterion of $|\log_2 \text{fold change}| > 1$ and a *p*-value <0.05. The enrichment analysis was performed using the KOBAS database and ClusterProfiler software to statistically evaluate the enrichment DEGs.

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

Gene expression at the transcript level was compared between wild-type males (ctrl), *cyp17a1*^{-/-};*cyp17a2*^{+/+} males (*cyp17a1*^{-/-}), and *cyp17a1*^{-/-};*cyp17a2*^{-/-} males (dko) at 180 dah using RT-qPCR. Testicular tissues were collected from each group (*n* = 3 per group). A total of 1.0 µg RNA was extracted from each sample and reverse transcribed using the Prime Script RT Master Mix Perfect Real Time Kit (Takara, Otsu, Japan), according to the manufacturer's instructions. RT-qPCR was then conducted in a 20 µL reaction mixture using an ABI-7500 Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, USA). The reaction setup included 10 µL of 2 × SYBR Premix ExTaq (Takara), 2 µL of diluted cDNA (with PCR-grade water used as a negative control), 6 µL of PCR-grade water, and 1 µL of each primer at 10 µmol/L concentration. The PCR protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 30 s. Melting curves were analyzed to assess primer specificity, and the relative gene expression levels were calculated using the ($R = 2^{-\Delta\Delta Ct}$) formula [19], with *β-actin* used as an internal control to confirm the efficiency of the reactions. The primer sequences used for PCR are listed in Supplementary Table S1.

2.9. Detection of apoptosis by TUNEL

The testes from wild-type males (ctrl), *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-};*cyp17a2*^{-/-} males (dko) at 6 months after hatching (mah) were initially fixed in 4 % paraformaldehyde (PFA) for 12 h at room temperature for the Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling (TUNEL) assay. After fixation, sectioning, and permeabilization, the tissues were processed to allow the TUNEL assay to detect apoptotic cells. TUNEL staining was performed using an In Situ Cell Death Detection Kit (Beyotime, Shanghai, China). In addition, the sections were stained with an antibody against Sycp3 (a marker for synaptonemal complex, 1:1000 dilution) followed by incubation with an Alex Fluor 488 conjugated secondary antibody (Invitrogen) to label the germ cells. The nuclei were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) to highlight cell nuclei. The images were captured using a confocal microscope (Olympus FV3000), allowing for the detailed visualization of apoptotic cells and germ cells within the tissue sections. The percentage of TUNEL-positive cells was quantified using ImageJ software, based on the total area of stained cells relative to

the total area within the images, as described in the above method.

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

Blood samples were drawn from the caudal veins of wild-type males (ctrl; *n* = 3), *cyp17a1*^{-/-};*cyp17a2*^{+/+} males (*cyp17a1*^{-/-}; *n* = 3), and *cyp17a1*^{-/-};*cyp17a2*^{-/-} males (dko; *n* = 3) following anesthesia (250 mg/L MS-222, Sigma, St Louis, MO, USA) at 180 days after hatching (dah) and were allowed to clot at room temperature for 1 h. The samples were then centrifuged at 3000g for 10 min, and the resulting supernatant was transferred to a fresh centrifuge tube. This supernatant was further centrifuged at 12,000g for 10 min at 4 °C and then stored at -80 °C until analysis. Serum levels of 11-keto-testosterone (11-KT) (582751), corticosterone (501320), and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (498500, 498502, and 498504) were quantified using ELISA kits (Cayman, Ann Arbor, MI, USA) as per the manufacturer's protocols. Absorbance was measured at a wavelength of 412 nm with a Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Additionally, to further assess the impact of the double mutation in *cyp17a1* and *cyp17a2* gene on steroidogenesis, Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) was employed to measure serum steroid levels using the AB Sciex QTRAP® 6500 LC-MS/MS system (AB Sciex, Framingham, MA, USA), with steroid details provided in Supplementary Table S2.

2.11. Statistical analysis

Data were analyzed using GraphPad Prism 8.2.1 software and presented as the mean ± SD (*n* = 3). Statistical significance of differences between groups was assessed using one-way ANOVA followed by the Tukey's test for multiple comparisons performed with SPSS software version 16.0. A *p*-value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Establishment of homozygous double mutants of *cyp17a1* and *cyp17a2* genes

The enzymes Cyp17a1 and Cyp17a2 are critical for steroidogenesis in tilapia, each playing unique roles in steroid hormone synthesis. Cyp17a1 possesses both 17α-hydroxylase and 17,20 lyase activities, facilitating the production of estrogens and androgens, whereas, Cyp17a2 only exhibits 17α-hydroxylase activity, primarily aiding in the synthesis of progestins and corticosteroids (Fig. 1A). Previous studies, including our own, developed single-gene mutants for *cyp17a1* and *cyp17a2* in Nile tilapia to explore their impact on reproductive processes [14,15]. To further explore their physiological roles, we created a double mutant line by crossbreeding heterozygous *cyp17a1* (+/-) and *cyp17a2* (+/-) individuals (Fig. 1B). Genotyping analyses were using PCR and Sanger sequencing, which revealed a 4-bp deletion in the *cyp17a1* gene and an 11-bp deletion in the *cyp17a2* gene in the double knockout (dko) fish (Fig. 1C). Compared to wild-type males (ctrl), mutations in the *cyp17a1* gene (*cyp17a1*^{-/-}) and the combined mutation of *cyp17a1* and *cyp17a2* (dko) resulted in an all-male phenotype, with males exhibiting gonadal hypotrophy, as evidenced by a reduction in the gonadosomatic index (GSI) (Fig. 1D, E), and led to male sterility, as indicated by the inability to obtain semen through abdominal compression (data not shown). Additionally, both *cyp17a1*^{-/-} males and *cyp17a1*^{-/-};*cyp17a2*^{-/-} males displayed disruptions in the structure of seminiferous lobules, abnormal proliferation of Leydig cells, and reduced sperm count in the efferent ducts, as confirmed by histological analysis and IF staining of the steroidogenic enzyme StAR in Leydig cells (Fig. 2).

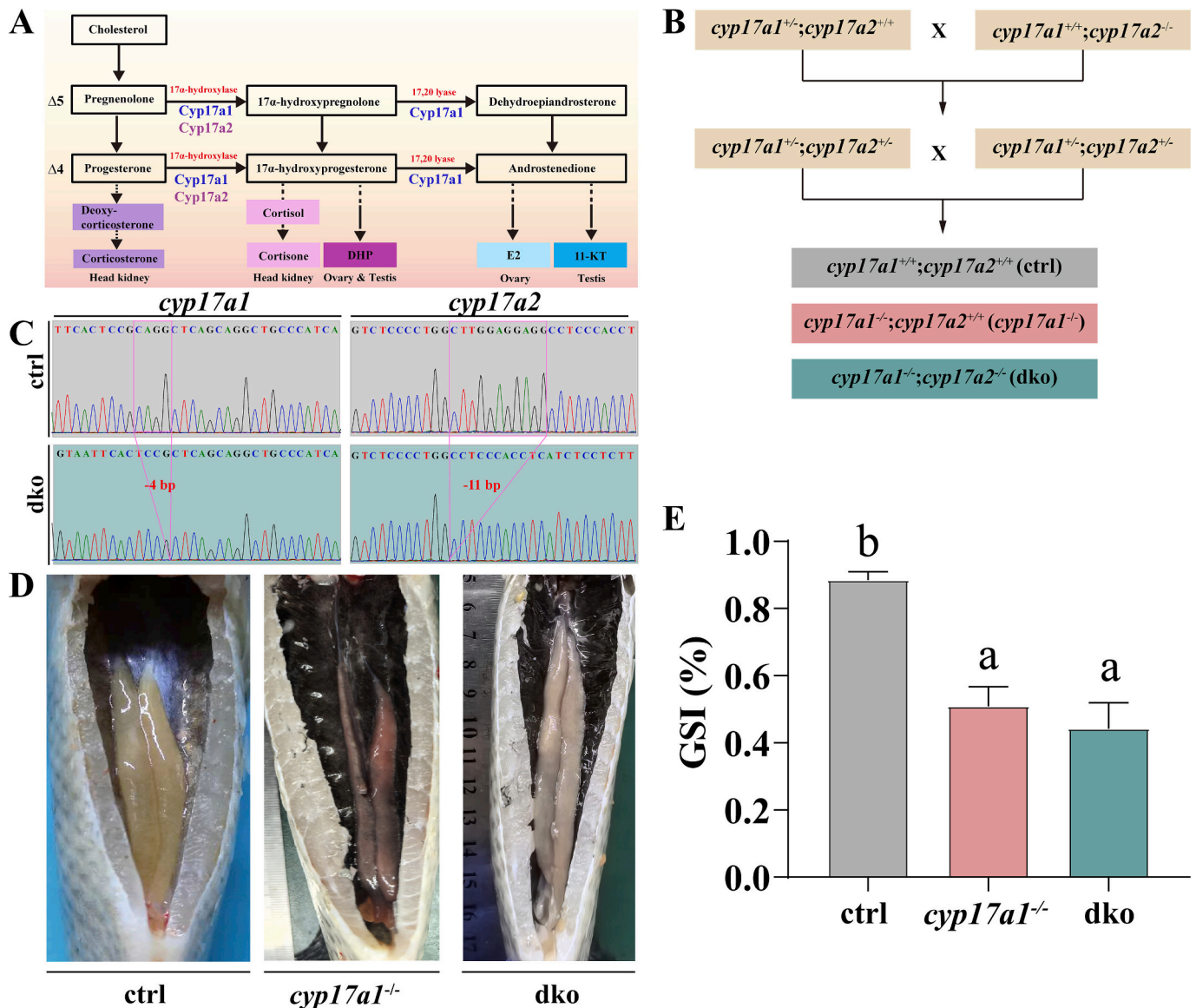


Fig. 1. Double mutant construction of *cyp17a1* and *cyp17a2* gene and gonadal development assessment.

A, Brief overview of the fish steroid hormone synthesis pathway, emphasizing the distinct enzyme activities and differences in steroid hormone production between Cyp17a1 and Cyp17a2. B, Schematic representation of how double mutants were created using heterozygous single gene mutants of *cyp17a1* and *cyp17a2*. C, The genotype of the double knockout (dco) fish, identified through Sanger sequencing analysis, revealed an indel with a 4-bp deletion in the *cyp17a1* gene and an 11-bp deletion in the *cyp17a2* gene in the dco fish. D, Anatomical analysis of the testes from wild-type (ctrl) males (a), *cyp17a1*^{-/-} males (b), and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dco) males (c) at 6 months post-hatching (mph). E, Comparison of the Gonadosomatic Index (GSI) among wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dco) males at 6 mph. Data are presented as mean ± SD. Different letters above the error bars denote significant differences as determined by one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$). GSI, Gonadosomatic index.

3.2. Genome-wide identification of RNAs

Transcriptome analysis was carried out on testes from wild-type males (ctrl), *cyp17a1*^{-/-}; *cyp17a2*^{+/-} males (*cyp17a1*^{-/-}), and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} males (dco) to investigate the potential roles of *cyp17a1* and *cyp17a2* in regulating spermiogenesis and male fertility. Following data cleaning, the RNA sequencing yielded between 20,561,198 and 29,959,810 clean reads per sample with Q30 values exceeding 94.53 % for each (Supplementary Table S3). Alignment to the tilapia reference genome was successful for 84.83 to 92.07 % of the reads, identifying a total of 17,911 genes (Supplementary Table S4). Analysis of these genes across the nine testicular transcriptomes revealed that 16,714, 16,248, and 16,286 genes were present in ctrl males, *cyp17a1*^{-/-} males, and dco males, respectively. Out of these, 15,070 genes were co-expressed across all three groups, while 898, 351,

and 395 genes were specifically expressed in ctrl males, *cyp17a1*^{-/-}, and dco males, respectively (Fig. 3A and Supplementary Table S5). A correlation heatmap indicated that correlation coefficients among different biological replicates ranged from 0.903 to 0.983, with significant distinctions noted between ctrl males and both *cyp17a1*^{-/-} and dco males, though differences between *cyp17a1*^{-/-} and dco males were relatively minor (Fig. 3B and Supplementary Table S6). Additionally, KEGG pathway enrichment analysis on the group-specific genes showed distinct functional annotations. Genes specifically expressed in the ctrl group were enriched in pathways like "Vascular smooth muscle contraction", "PPAR signaling pathway", and "Carbon metabolism"; those in the *cyp17a1*^{-/-} group were enriched in the "Pyrimidine metabolism", "Apoptosis - multiple species", and "Acute myeloid leukemia" pathways; and genes in the dco group were primarily associated with "Primary immunodeficiency", "Cell adhesion molecules", and "Hippo

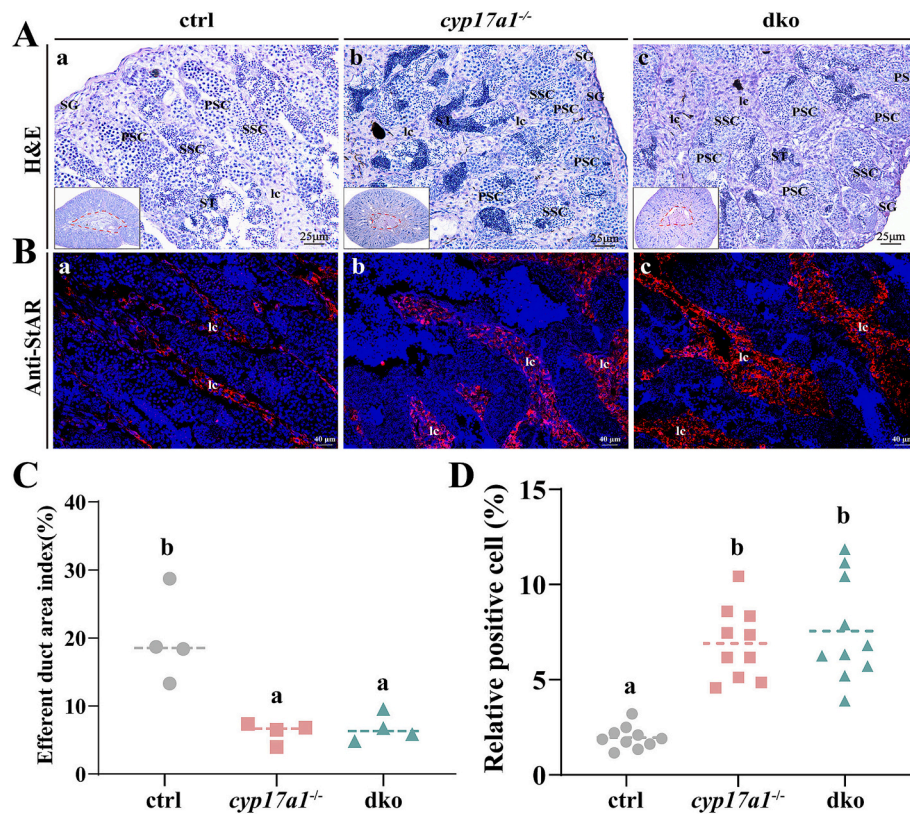


Fig. 2. Abnormal proliferation of Leydig cells caused by the deficiency of *cyp17a* genes.

A, Histological observation of testes from wild-type (ctrl) males (a), *cyp17a1*^{-/-} males (b), and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males (c). The red dotted lines highlight the efferent ducts. B, Immunofluorescence staining of StAR in the testes of wild-type (a), *cyp17a1*^{-/-} males (b), and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males (c). Red and blue signals represent positive StAR and DAPI staining, respectively. Key cellular markers identified include spermatogonia (SG); primary spermatocytes (PSC); secondary spermatocytes (SSC); spermatids (ST); Leydig cells (lc). C, Comparative analysis of the area percentage of the efferent duct in wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males. D, Quantification of the area percentage of the StAR-positive cells in the testes of wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males. Data are presented as mean ± SD. Distinct letters above the error bars signify statistical differences, determined by one-way ANOVA followed by Tukey's post hoc test (*P* < 0.05).

signaling pathway-multiple species" (Fig. 3C-E).

3.3. *Cyp17a1* deficiency led to an increased apoptosis of germ cells

As shown in Fig. 3D, the genes uniquely expressed in *cyp17a1*^{-/-} males were predominantly enriched in the "Apoptosis – multiple species" pathway. Therefore, cell apoptosis was assessed in the testes of wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males. The results showed that an increase in positive TUNEL staining was most prominently observed in the primary spermatocytes (Sycp3+ cells) of *cyp17a1*^{-/-} males and dko males at 6 mah (Fig. 4).

3.4. Analysis of global differentially expressed genes (DEGs)

Genes with an absolute log2 fold change of ≥1 and an FDR < 0.05 were classified as DEGs. The Venn diagram in Fig. 6A illustrates that a total of 2688 DEGs were identified across the three libraries. Specifically, the overlaps of DEGs between ctrl vs. *cyp17a1*^{-/-}, ctrl vs. dko, *cyp17a1*^{-/-} vs. dko were 1000, 86, and 101, respectively (Fig. 5A and Supplementary Table S7). A heatmap displaying the expression patterns of these 2688 DEGs across all three groups showed that most genes in both *cyp17a1*^{-/-} and dko testes exhibited similar expression trends, with genes downregulated in the testes of *cyp17a1*^{-/-} and dko males being upregulated in the testes of ctrl males (Fig. 5B). When comparing with ctrl males, 1713 DEGs were found in *cyp17a1*^{-/-} males and 1799 DEGs in dko males, with 662 and 797 genes upregulated, and 1051 and 1002 genes downregulated, respectively (Fig. 5C, D). Between

cyp17a1^{-/-} and dko males, there were 351 DEGs, including 258 upregulated and 93 downregulated genes (Fig. 5E).

3.5. Kyoto encyclopedia of genes and genomes enrichment analysis of differentially expressed genes

In this study, KEGG, a public database related to biological pathways, was utilized to analyze the biological pathways of DEGs. Specifically, 190 pathways were found to be enriched in the comparison between wild-type males (ctrl) and *cyp17a1*^{-/-} males, as listed in Supplementary Table S8, with the top 20 pathways illustrated in Fig. 6A. these pathways include "Primary bile acid biosynthesis", "ABC transporters", "PPAR signaling pathway", and "Thyroid hormone synthesis". For the comparison between ctrl and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} males (dko), 185 pathways were enriched which are detailed in Supplementary Table S8 and include pathways like "Alanine, aspartate and glutamate metabolism" as well as well-known metabolic pathways such as "Primary bile acid biosynthesis" and "ABC transporters". Notably, pathways such as "steroid hormone biosynthesis", "Thyroid hormone synthesis" and "metabolism of xenobiotics by cytochrome P450" were also highlighted (Fig. 6B). In comparison between *cyp17a1*^{-/-} and dko males, 97 pathways were enriched, including "Hepatitis C", "Cytokine-cytokine receptor interaction", "Steroid hormone biosynthesis", "HIF-1 signaling pathway", "PPAR signaling pathway", and "Biosynthesis of unsaturated fatty acids" (Fig. 6C). Additionally, the expression patterns and differences of several genes involved in "Primary bile acid biosynthesis", "ABC transporters", and "Alanine, aspartate and glutamate

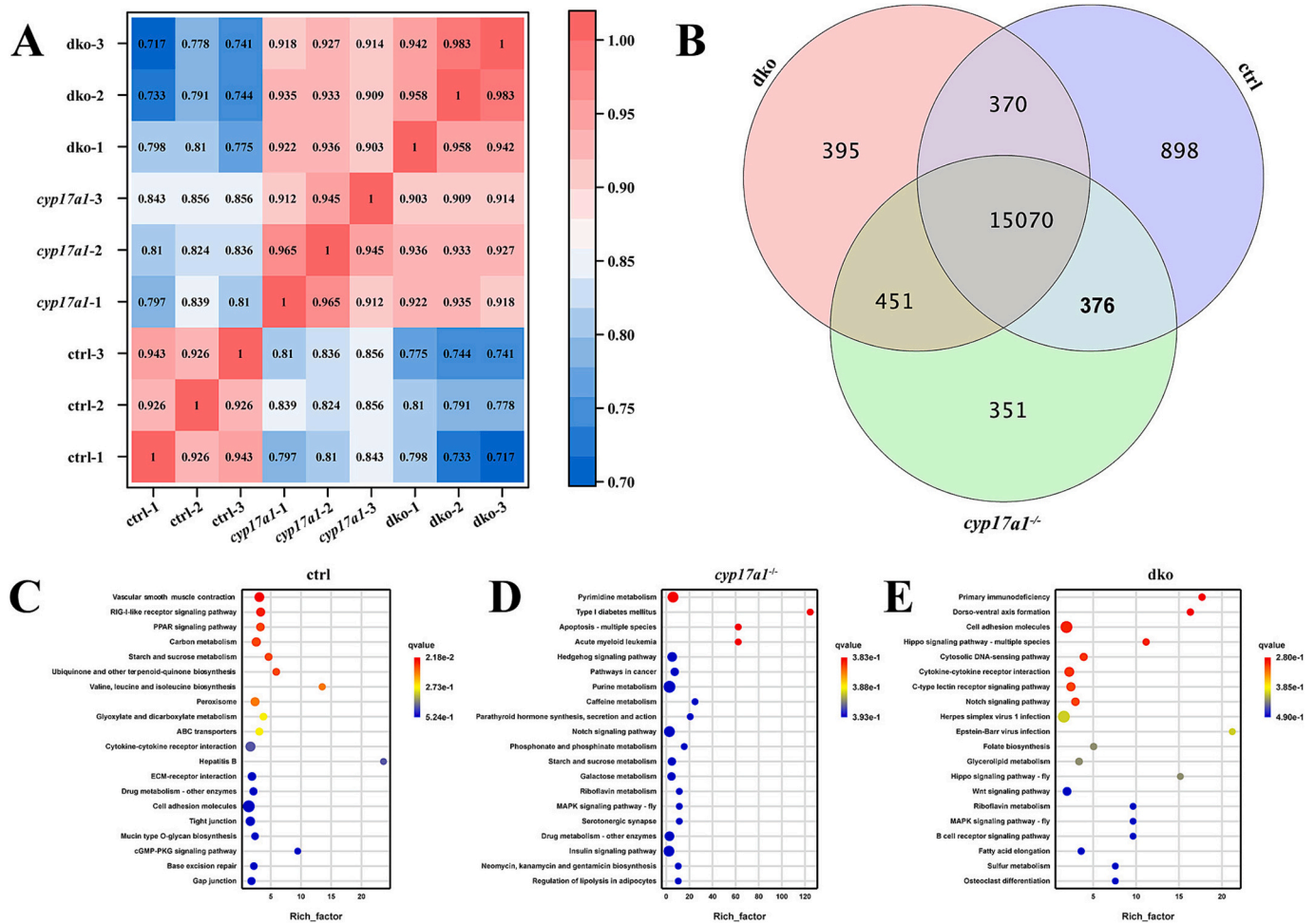


Fig. 3. Overview of gonadal transcriptome.

A, The correlation heatmap illustrates the relevance coefficient based on the gene expression patterns among wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males, calculated using Pearson correlation coefficients. The heatmap uses a color gradient from red to blue to show the strength and direction of correlations, with a scale bar on the right indicating values from 0.70 to 1.00. B, A Venn diagram shows genes co-expressed and specific expressed among wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males. C-E, KEGG enrichment of specifically expressed genes in wild-type (ctrl) males (C), *cyp17a1*^{-/-} males (D), *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males (E). The horizontal axis of the plot shows the enrichment factor, while the vertical axis lists the top 20 enriched pathways. The color gradient from red to blue indicates the significance level (q-value), and the bubble size represents the number of sample-specific genes mapped to each pathway.

metabolism” were visualized using heatmaps and validated by RT-qPCR (Fig. 6D-H), providing an extensive view of gene expression variations linked to these significant pathways.

3.6. Insufficient sex steroid hormone levels in double mutants of *cyp17a1* and *cyp17a2* genes

KEGG pathway analysis revealed that the DEGs across the three groups were notably enriched in the “Steroid hormone biosynthesis” pathway (Fig. 6A-C). consequently, we examined the expression differences of key genes involved in steroidogenesis among the three groups, including *star1*, *star2*, *hsd11b2*, *sf-1*, *gth-ri*, *gth-rii*, *ara*, *arb*, and *pgr*, utilizing RT-qPCR (Fig. 7A and B). To further explore the impact of mutation in the *cyp17a1* and *cyp17a2* genes on steroidogenesis, we measured steroid hormone levels using ELISA and LC-MS/MS. Results indicated that mutations in the *cyp17a1* gene in male tilapia led to decreased levels of androgens (androstenedione, dehydroepiandrosterone, testosterone, and 11-ketotestosterone) and estrogens (estrone and 17 β -estradiol). Conversely, mutations in the *cyp17a2* gene hindered the production of progestins (DHP) and corticosteroids (cortisol and cortisone). Interestingly, mutations in the *cyp17a2* gene also resulted in an

atypical increase in the production of progestins (progesterone, pregnanediol), estrogens (2-methoxy-estrone and 4-methoxy-estrone), and corticosteroids (deoxycorticosterone and corticosterone) (Fig. 7C-F and Supplementary Table S9).

4. Discussion

Estrogens, particularly 17 β -estradiol, are considered one of the most important factors in female sex differentiation and maintenance in fish [2,20–23]. *Cyp17a1*, as a key enzyme in the estrogen synthesis pathway, facilitates the conversions of upstream substrates (pregnenolone and progesterone) into estrogen precursors (dehydroepiandrosterone and androstenedione) by catalyzing 17 α -hydroxylase and 17,20 lyase activities [7,8,12]. Mutation of the *cyp17a1* gene resulted in female-to-male sex reversal due to the impaired estrogen synthesis, whereas mutation of *cyp17a2* had no effect on sexual differentiation in either zebrafish or tilapia, indicating that *cyp17a2* does not play a significant role in estrogen biosynthesis [13,14,17,18]. While *cyp17a2*, the paralog of *cyp17a1* in fish, functions solely as a 17 α -hydroxylase, catalyzing the conversion of pregnenolone and progesterone to 17OH-pregnenolone and 17OH-progesterone, which are precursors for cortisol and DHP

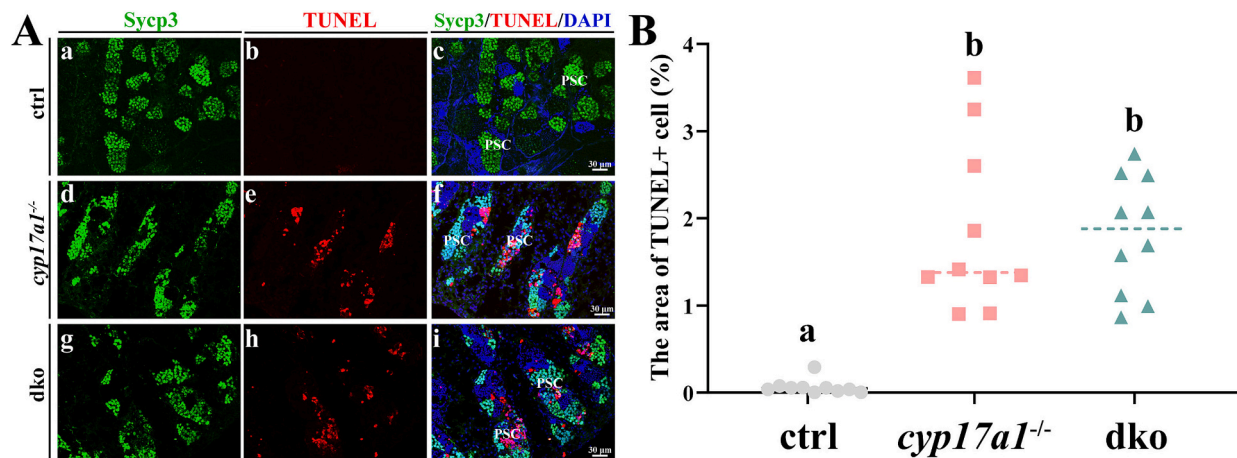


Fig. 4. Germ cell apoptosis induced by *cyp17a1* and double mutation of *cyp17a1* and *cyp17a2* genes in testes.

A, Apoptotic spermatogenic cells in wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-};*cyp17a2*^{-/-} (dko) males were detected by TUNEL staining and immunofluorescence (IF) staining of Sycp3. Red, green, and blue fluorescence represent the TUNEL, Sycp3, and DAPI signals, respectively. PSC, primary spermatocytes. B, Percentage area of positive TUNEL staining in wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-};*cyp17a2*^{-/-} (dko) males. The data are reported as the mean ± SD. Different letters above the error bar indicate statistical differences, which were tested by one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).

synthesis [7,8,12]. Although the roles of Cyp17a1 and Cyp17a2 in male and female reproduction have been investigated using single gene mutation in tilapia and zebrafish, their specific function remain an area of ongoing research. Therefore, both single and double gene mutations of *cyp17a1* and *cyp17a2* were generated, and the landscape of serum steroid levels and transcriptome analysis were conducted to investigate the widespread molecular changes in the testes.

Previous studies have highlighted the distinct enzyme activities of Cyp17a1 and Cyp17a2, with both sharing 17 α -hydroxylase activity and showing varying affinities for substrates [7,12]. While, both Cyp17a enzymes exhibit similar progesterone 17 α -hydroxylation activity, Cyp17a2, with its higher *K_m* values, catalyzes pregnenolone 17 α -hydroxylation more effectively than Cyp17a1. In addition, Cyp17a1 exhibits 17 α , 20-lyase activities and catalyzes the progesterone 17 α , 20-lyase reaction more efficiently than the pregnenolone 17 α , 20-lyase reaction [12]. In the present study, mutating *cyp17a1* in *cyp17a2* mutant background resulted in an all-male phenotype due to estrogen deficiency, further highlighting the essential role of estrogens in female sexual differentiation. In addition, mutations in the *cyp17a1* gene alone and combined mutations in both the *cyp17a1* and *cyp17a2* genes led to reduced levels of androgens (androstenedione, dehydroepiandrosterone, testosterone, and 11-ketotestosterone). However, the mutations in *cyp17a1* alone did not impact the production of progestins (including 17 α -hydroxypregnenolone, progesterone, and DHP) or corticosteroids (including cortisol, cortisone, deoxycorticosterone, and corticosterone). In contrast, the double mutation in both *cyp17a1* and *cyp17a2* in tilapia resulted in a significant decrease in DHP, cortisol, and cortisone, along with an abnormal increase in pregnanediol, deoxycorticosterone, and corticosterone production. Therefore, these results further emphasized the distinct substrate preferences of fish Cyp17a enzymes, with these preferences led to the paralogue-specific steroid pathways.

Furthermore, males with the dko exhibited phenotypes similar to those in *cyp17a1*^{-/-} but differed from those in *cyp17a2*^{-/-} males, characterized by smaller testes and efferent duct, abnormal proliferation of Leydig cells, and absence of semen production, primarily due to androgen deficiency resulting from the *cyp17a1* mutation, with androgen serving as a critical endocrine regulator of male testicular development and fertility in fish [14,15,24,25]. These findings highlight the critical role of androgens in testicular development and male fertility, and demonstrate that C21-class steroids serve as complements to androgens in the reproductive processes of male fish. Unexpectedly, the levels of endogenous estrone metabolites, 2-methoxy-estrone and 4-

methoxy-estrone, were abnormally elevated in dko males. This suggests that disruption of steroidogenesis due to the mutation of *cyp17a1* and *cyp17a2* genes resulted in steroid homeostasis imbalance, led to the generation of alternative metabolites through atypical steroid synthesis pathways.

Further, this study extensively analyzed gene expression patterns in the testes of ctrl, *cyp17a1*^{-/-}, and dko males, identifying a total of 17,911 genes. Among these, 15,070 genes were co-expressed across all three groups, while 898, 351, and 395 genes were specifically expressed in ctrl, *cyp17a1*^{-/-}, and dko males, respectively. Pathway enrichment analysis revealed that genes involved in the "Apoptosis" and "Hedgehog signaling pathway" pathways were notably expressed in the testes of *cyp17a1*^{-/-} males. Hedgehog proteins, which act as morphogens that drive cell proliferation and development in vertebrates [26–30]. Activation of Hedgehog signaling has been shown to increase the proliferation of Leydig cells and spermatogonia, whereas its inhibition has been associated with an increase in germ cell numbers and a suppression of primordial germ cells [26,29–31]. In mice, the absence of Leydig cells and a block in germ cell development leading to male infertility were observed in null mutant males of the *Dhh* gene, underlining the critical role of Hedgehog signaling in Leydig cell proliferation and male fertility [32]. Additionally, the notch signaling pathway also plays crucial roles in the development and function of both male and female reproductive systems [33]. Thus, it is suggested that these genes, enriched in these pathways, may contribute to the increased apoptosis of germ cells, abnormal proliferation of Leydig cells, and the infertility observed in *cyp17a1*-deficient males.

The DEG analysis of testes from ctrl, *cyp17a1*^{-/-}, and dko males identified a total of 2688 DEGs across the three comparison. Specifically, 1731 DEGs (662 upregulated, 1051 downregulated), were found in ctrl vs. *cyp17a1*^{-/-}, 1799 DEGs (797 upregulated, 1002 downregulated) in ctrl vs. dko, and 351 DEGs (258 upregulated, 93 downregulated) in *cyp17a1*^{-/-} vs. dko. KEGG pathway enrichment analysis revealed that the DEGs in both ctrl vs. *cyp17a1*^{-/-}, and ctrl vs. dko were predominantly associated with pathways such as "Primary bile acid biosynthesis", "ABC transporters", "Thyroid hormone synthesis", and "Alanine, aspartate and glutamate metabolism".

Bile acids are synthesized in the liver from cholesterol and are known to signal through two receptors: the nuclear Farnesoid-X-Receptor alpha (FXR α ; NR1H4) and the membrane receptor TGR5 (GPBAR1, G protein-coupled bile acid receptor) [34]. In mice, activation of FXR α signaling in Leydig cells decreased the expression of steroidogenesis factor-1 (*sf-1*),

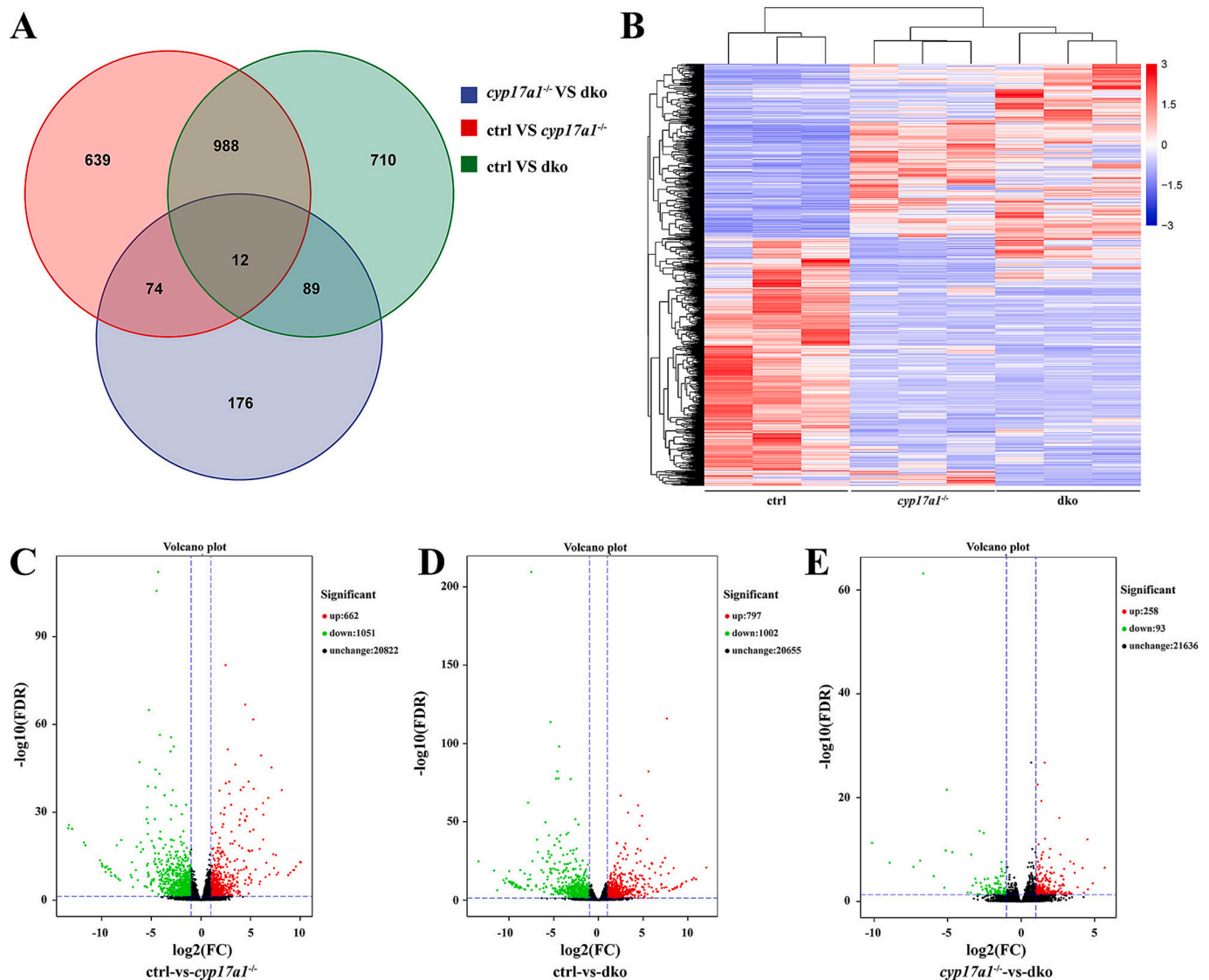


Fig. 5. General overview of the DEGs across the gonadal transcriptome

A, Venn diagram displays the gonadal DEGs identified across three comparisons in Nile tilapia, illustrating the overlap and unique expression patterns. B, Heatmap of DEGs among wild-type (ctrl) males, *cyp17a1*^{-/-} males, *cyp17a1*^{-/-};*cyp17a2*^{-/-} (dko) males based on the gene expression patterns. The color scale transitions from red, indicating upregulation, to blue, signifying downregulation. C-E, Volcano plots highlight the DEGs in the testes when comparing wild-type (ctrl) males with *cyp17a1*^{-/-} males (C), wild-type (ctrl) males with *cyp17a1*^{-/-};*cyp17a2*^{-/-} (dko) males (D), and *cyp17a1*^{-/-} males with *cyp17a1*^{-/-};*cyp17a2*^{-/-} (dko) males (E). Significantly differentially expressed genes are marked in red for upregulation and blue for downregulation, while genes without significant changes are shown in black. The x-axis represents the log₂ fold change, and the y-axis represents the -log₁₀(FDR).

which known to activate the expression of genes encoding steroidogenic enzymes, as well as the steroidogenic enzymes, including *star*, *cyp11a1*, and *hsd3b1*, and resulted in a decline in androgen production independently of the hypothalamic-pituitary axis [35,36]. Feeding bile acid diet to adolescent mice led to reduced testosterone synthesis, delayed sexual maturation, decreased sperm production, and impaired fertility. However, supplementation with testosterone counteracted the effect of bile acid diet [37]. Similarly, sex hormones are gradually being recognized as important regulators of bile acid production through critical hepatic feedback mechanisms. Estrogens and androgens regulate cholesterol and energy homeostasis by binding to their receptors and influence the rate-limiting step of bile acid synthesis from cholesterol by modulating the expression of *Cyp7a1* in the liver [38,39]. Combined with the disruption of steroid synthesis caused by genetic mutations, single gene mutation in *cyp17a1* and double mutations in *cyp17a1* and *cyp17a2* altered the expression of genes associated with bile acid biosynthesis. We propose that the steroids catalyzed by *Cyp17a1* and *Cyp17a2* play a

key role in regulating bile acid synthesis in the liver of fish.

ATP-binding cassette (ABC) transporters, a family of transmembrane proteins that have been suggested as modulators of steroidogenesis, fertilization, fetal development, and immune responses. ABC transporters play a crucial role in transmembrane transport of steroid hormones (including estrogens, androgens, progestins, and corticosteroids) and their precursors, while also regulating the movement of compounds involved in inflammatory and immunological responses [40]. In addition, ABC transporters serve as “gatekeepers” of the blood-testis barrier, which is maintained by tight junctions between Sertoli cells within the seminiferous tubules to protect the testes from potentially harmful xenobiotic factors [41]. Moreover, in mice, *Abca1* deficiency resulted in a lipid accumulation in Sertoli cells and significantly reduced the intratesticular testosterone levels and sperm counts [42]. In this study, about 14 genes associated to ABC transporters pathway were significantly altered both in *cyp17a1*^{-/-} and dko males, with 12 genes showing significant upregulation in the mutants. These findings suggest that

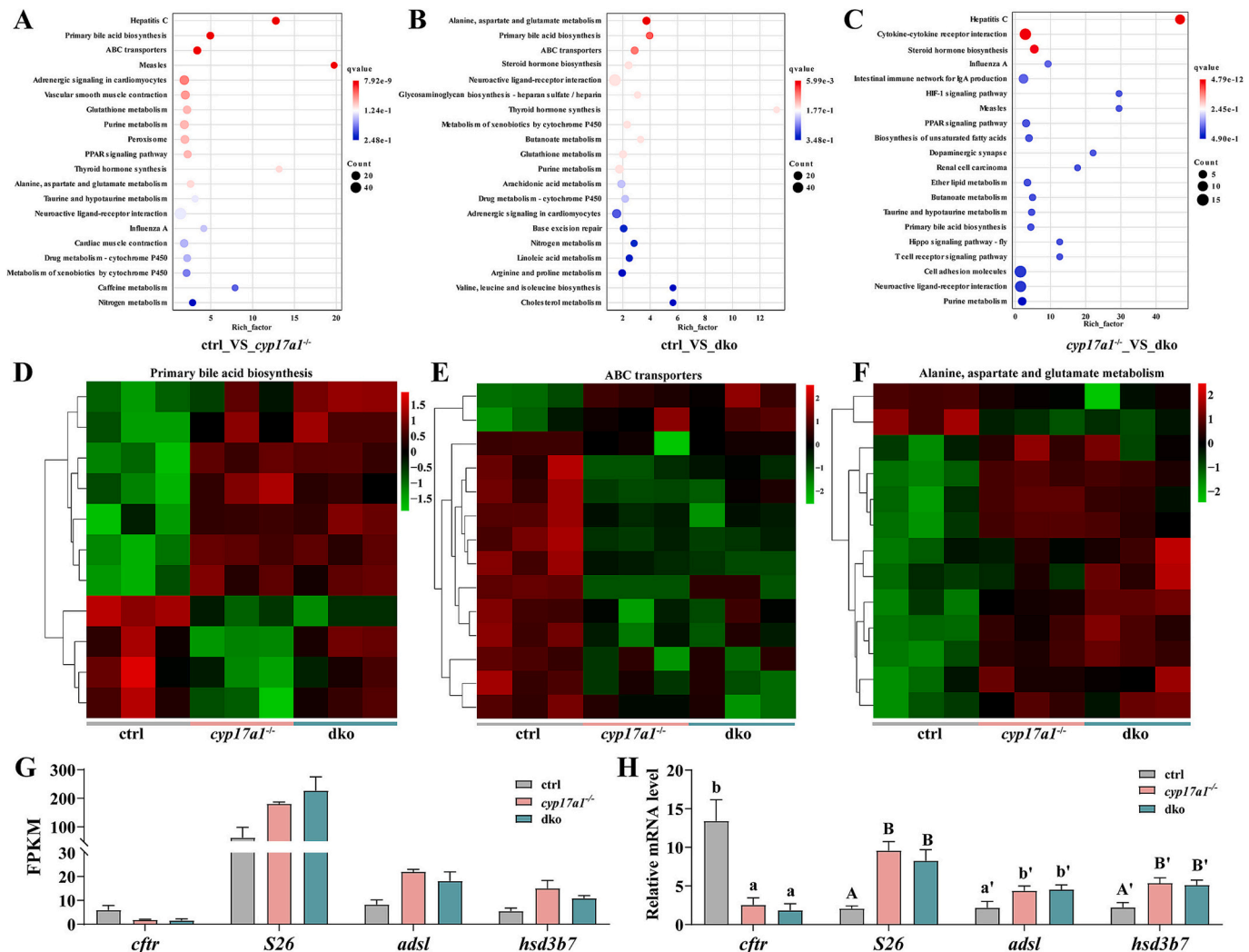


Fig. 6. KEGG enrichment analysis of DEGs.

A-C, Bubble diagram of the top 20 enriched KEGG pathways for DEGs screened from wild-type (ctrl) males vs *cyp17a1*^{-/-} males (A), wild-type (ctrl) males vs *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males (B), and *cyp17a1*^{-/-} vs *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males (C). The enrichment factor, displayed along the x-axis, represents the ratio of the number of DEGs to the total number of within a pathway. The size and color of each bubble indicate the number of genes and the range of q-values, respectively, highlighting the significance of the pathway enrichment. D-E, Heatmap showing the expression patterns of DEGs identified through KEGG enrichment analysis across wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males. The color gradient ranges from high expression (red) to low expression (green), providing a visual representation of gene activity differences among the groups. G, FPKM indicates transcript level of selected genes according to the RNA-seq data. H, RT-qPCR based transcriptional profiling of these selected genes in the testes of wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males. β -actin was used as the reference gene for normalizing the expression values obtained via qRT-PCR. The data are reported as the mean ± SD. Different letters above the error bar for each gene indicate statistical differences, which were tested by one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).

mutation in *cyp17a* may alter the expression of ABC transporters, thereby influencing transmembrane transport processes and blood-testes barrier formation, both of which are critical for spermatogenesis and male fertility.

Additionally, the comparison between *cyp17a1*^{-/-} and dko males revealed distinct enrichment of DEGs in pathways, such as “HIF-1 signaling pathway” and “Hippo signaling pathway”. Hippo signaling, an evolutionarily conserved network, regulates cell proliferation [43]. Steroid hormones (e.g. testosterone and estradiol) enhanced the expression and activity of its downstream effectors, while inhibition of Hippo signaling via pharmacological methods or gene silencing suppressed the production of estradiol and progesterone [44–47]. And the HIF-1 signaling, which serves as a major molecular mediator of the hypoxic response [48]. Previous studies have demonstrated that HIF-1 functions as a down-regulator of the progesterone receptor (PGR), mutation of the PGR gene in mice led to a decline expression of HIFs [49,50]. In our study, the double mutation of *cyp17a1* and *cyp17a2*

genes but not the single mutation of *cyp17a1* gene, led to the accumulation of progesterone and a decline in DHP, which could potentially lead to the changes in the expression of genes associated with the HIF-1 and Hippo signaling pathways, suggesting that *cyp17a1* and *cyp17a2* mutations contribute to unique molecular differences, potentially influencing immune signaling and metabolic regulation [51,52].

This study comprehensively explored the combined effects of *cyp17a1* and *cyp17a2* on steroidogenesis and male fertility in Nile tilapia, utilizing both single and double mutant models for comparative analysis. The results demonstrated that Cyp17a isoforms play crucial roles in testicular development and male fertility. They maintain steroidogenic accuracy through substrate-specific enzymatic actions and influence various signaling pathways that regulate metabolic adjustments, immune responses, and germ cell viability when steroidogenesis is disrupted. These findings offer detailed mechanism underlying male infertility associated with defects in steroid biosynthesis.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.144772>.

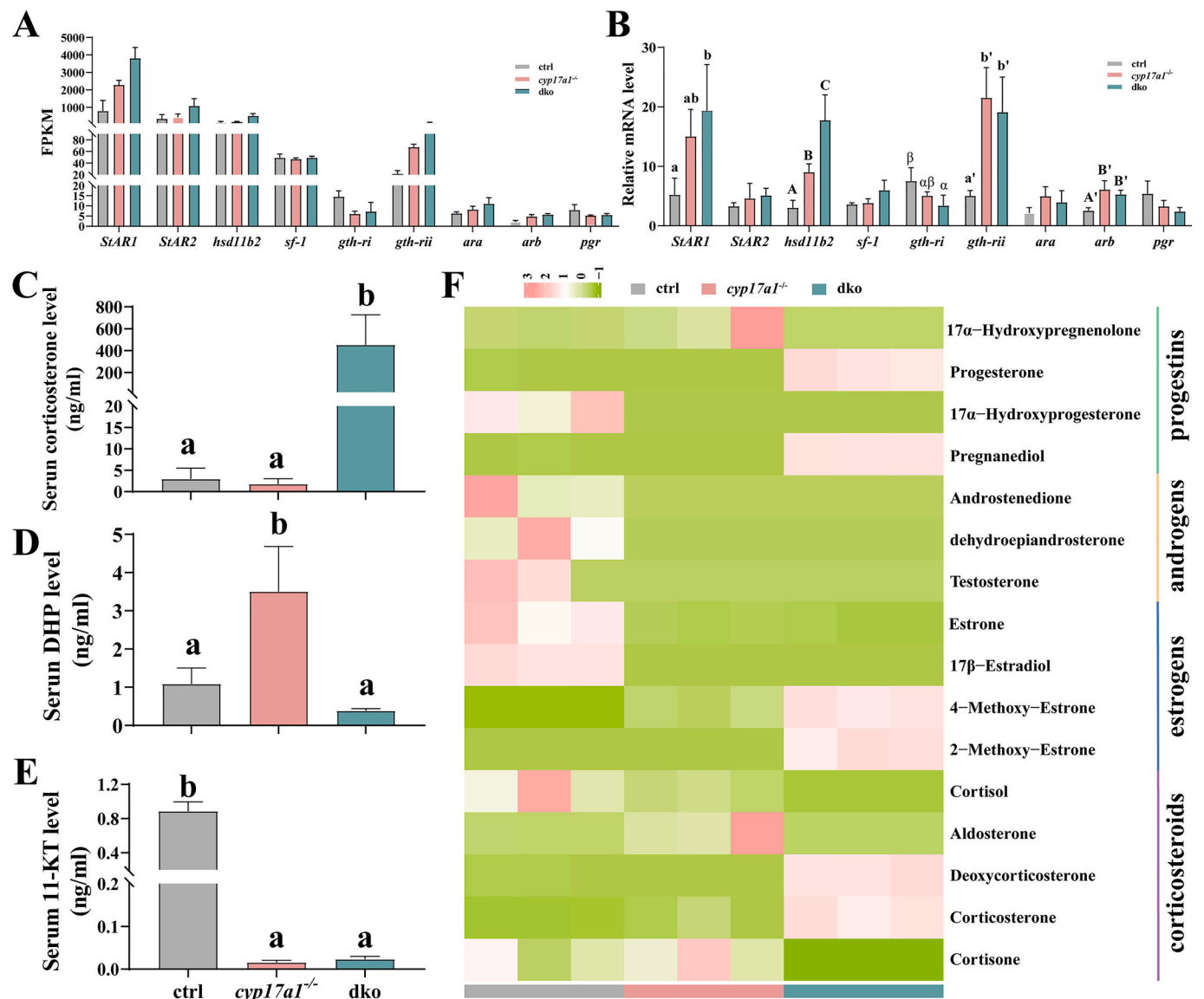


Fig. 7. Landscape of steroid levels in double mutants of the *cyp17a1* and *cyp17a2* genes. A, FPKM indicates transcript level of various genes according to the RNA-seq data. B, Gene expression levels of in the testes of wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males quantified using qRT-PCR. *β -actin* was used as the reference gene for normalizing the expression values obtained via qRT-PCR. C-E, Levels of serum corticosterone, DHP, and 11-KT levels in wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males, assessed using an ELISA kit. F, Serum steroid concentrations in wild-type (ctrl) males, *cyp17a1*^{-/-} males, and *cyp17a1*^{-/-}; *cyp17a2*^{-/-} (dko) males, measured by LC-MS/MS. Data for qRT-PCR and steroid levels are presented as means $\pm$ SD. Different letters above the error bar for each gene indicate statistical differences, which were tested by one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).

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CRedit authorship contribution statement

Lanyang Yang: Writing – original draft, Visualization, Validation, Software, Methodology, Funding acquisition. **You Wu:** Validation, Methodology, Investigation. **Yiyun Du:** Visualization, Validation, Software, Methodology. **Maolin Ye:** Formal analysis, Data curation. **Yanbin Zhang:** Formal analysis, Data curation. **Deshou Wang:** Writing – review & editing, Supervision, Conceptualization. **Linyan Zhou:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are presented in the article and Supplementary materials.

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