Histological and sex-determining genes expression effects of 17 α -methyltestosterone on mandarin fish *Siniperca chuatsi* gonad development

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Research article

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Abstract

Background

17 α -methyltestosterone (MT) as a synthetic androgen has been widely used in sex reversal of fish research, but there was no published report on sex reversal of mandarin fish by MT. Moreover, comparative transcriptome analysis of testis and ovarian tissue is still lacking in mandarin fish. We used histological analyses together with RNA sequencing to characterize mandarin fish gonadal transcriptomes and investigate the effects of MT on the sex ratio, survival rate, growth, gonadal differentiation.

Results

Mandarin fish treated with dietary MT at 50 mg/kg and 100 mg/kg dosages were successfully induced to all-male stock (male rate 100%), compared with the control group (51.11%). The survival rate of fish in the MT treated and control groups were not significant different. MT were significantly inhibited the growth of the MT treatment group ($P < 0.05$) at the 20 dph-120 dph, however, the weight and length in the MT treated and control groups were not significant different at the 180 dph and 240 dph. MT treatment promoted the development of testis, but inhibited the gonadosomatic index (GSI) and the levels of serum steroid hormone (T and E₂). This work screened out the genes related to the sex determination and differentiation of the fish by sequencing and analysis of the transcriptome of the ovary and testis. The masculinization of mandarin fish was also demonstrated by the expression patterns of sex-specific genes, *dmrt1*, *sox9*, *foxl2* and *cyp19a1a*: the gonads of MT-treated fish exclusively expressed male-specific *dmrt1* and *sox9* with no expression of female-specific *foxl2* and *cyp19a1a*.

Conclusion

This study suggests that 17 α -methyltestosterone successfully induced all-male stock and we select the part of the genes (*dmrt1*, *sox9*, *foxl2* and *cyp19a1a*) related to sex determination and differentiation.

Background

The mandarin fish (*Siniperca chuatsi*) is an economically important fish in China. The growth of mandarin fish shows gender dimorphism that the growth rate of female was over 15% higher than that of males [1]. Therefore, the production of all-female stock of mandarin fish can provide important technical support for the cultivation of excellent growth character and economic character. The first step to produce all-female of mandarin fish is artificially induced sex reversal. The synthetic steroid 17 α -methyltestosterone (MT) is a male-specific hormone commonly used to induce sex reversal in teleost fish [2]. The effect of MT is dependent on various factors such as timing, dose and duration of treatment [3]. MT was successfully used to masculinize genetic females into phenotypic males, such sex reversed females can produce X-type spermatozoa whose offspring would be 100% females, and the offspring can be used for actual production [4, 5]. To our knowledge, the application of MT has been reported to be effective in some species such as zebrafish (*Danio rerio*) [6], Nile tilapia (*Oreochromis niloticus*) [7], orange-spotted grouper (*Epinephelus coioides*) [8] and yellow catfish (*Pelteobagrus fulvidraco*) [9], but MT has been reported to have negative effects on survival, growth and/or gonadal development if a particular threshold dose is surpassed [10, 11]. Therefore, the dose, timing and duration of MT used for induction of sex reversal is species-specific, and the effects of androgens need to be clarified in mandarin fish. In order to prove whether MT can successfully induce sex reversal mandarin fish to produce all-female populations, we investigated the effects of MT on the sex ratio, survival rate and growth performance. Moreover, the gonadal development of mandarin fish was determined by histological observation and levels of sex steroids testosterone (T) and 17 β -estradiol (E₂) in serum.

Recent studies have shown that RNA-seq is a powerful strategy to quantify gene expression levels [12]. RNA-Seq has been proven more suitable to quantify transcripts expressed at low levels than microarrays or EST analysis [12–14]. This is because RNA-seq verifies direct transcript profiling without compromise or bias. The advantages of RNA-seq make it widely used in transcriptome profiling studies in relation to gonadal development of various fish species, including spotted knifejaw (*Oplegnathus punctatus*) [15], Nile tilapia [16, 17] and fugu (*Takifugu rubripes*) [18]. However, comparative transcriptome analysis of testis and ovarian tissue is still lacking in mandarin fish.

Sex determination in fish encompasses both genetic and environmental sex determination, genetic sex determination refers to the gender of the offspring determined by the sex-determining genes on the chromosome and its interaction with the secondary genetic factors [19]. Nevertheless, few genes involved in gonadal differentiation have been identified in fish. Matsuda, et al. [20] reported that the gene named DMY (DM domain gene on Y-chromosome) was cloned from the recombination and deletion analysis of the sex-determining segment on the Y chromosome of medaka fish (*Oryzias latipes*). Functional analysis indicated that this gene plays an important role in sex determination of medaka fish. However, in other fish such as celebes medaka (*Oryzias celebensis*), rainbow trout (*Oncorhynchus mykiss*),

Nile tilapia, zebrafish, etc., the homologous sequence could not be found [21, 22]. Furthermore, sex-determining genes have yet to be discovered and identified in mandarin fish.

There are no studies with detail on sex reversal of mandarin fish by 17 α -methyltestosterone. This article focused on the effect of MT on the sex ratio, survival rate, growth performance and gonadal development of mandarin fish. Differential gene expression in female and male gonads were identified by using transcriptome sequencing technology. Characterization and expression profiles of sex-determining genes were examined during gonadal sex differentiation by MT induction in mandarin fish.

Results

Growth status and sex ratios

The body weight and body length of mandarin fish during MT treatment were shown in Table 1. The results showed that the body weight and body length of the MT-treated group were significantly lower than those of control group ($P < 0.05$) from 20 dpf to 70 dpf. Among the MT treatments, the body weight and body length of M1 group were higher than M2 group with no significant difference ($p > 0.05$). The body weight and body length of post-treatment were summarized in Fig.1 and Fig.2. At 120 dpf, the body weight and body length of the MT treatments were significantly lower than those of control group ($P < 0.05$). Nevertheless, no significant differences were found for the body weight and body length among the control group and MT treatments at 180 dpf and 240 dpf. There were also no significant differences in body weight and body length between the MT treatment groups at 120 dpf, 180 dpf and 240 dpf.

The survival rates of the MT treatments and control group were not significantly different (Fig.3). The MT-treated sex ratios were 100% males, while the control sex ratios were 51.11% males at 70dpf, and the ratio of females and males in the control group was about 1:1 (Table 2.).

Gonadal morphology and serum steroid hormone

At 70 dpf, the control males had testes which were populated with large volumes of spermatocytes and a few spermatogonia and spermatid and the MT-treated testes were consisted of large volumes of spermatid and a few spermatocytes and spermatogonia (Fig. 4 a, b, c). At 120 dpf, a few spermatozoa were shown in the control testes (Fig. 4 d), the SZ were strongly basophilic whose nuclei were stained dark blue by hematoxylin. In the seminiferous lobules away from the vas deferens, most of them were filled with spermatid. The maturity of MT-treated testes was significantly higher than that of the control testes, which testes were filled with spermatozoa (Fig. 4 e, f). At 180 dpf, in the seminiferous lobules closed to the vas deferens of the control testes were populated with a few spermatozoa. Moreover, the lobular lumen was reduced and the lobular wall was thickened, and spermatozoa in the lobular lumen were significantly reduced or disappeared. The lobular lumen of MT-treated testes also showed signs of degeneration, but large volumes of spermatozoa were still found in the seminiferous lobules closed to the vas deferens (Fig. 4 h, i). At 240 dpf, the lobular lumen of the MT-treated testes and the control testes were reduced to almost disappear, the lobular wall was thickened, and the lobular interstitial was developed. (Fig. 4 j, k, l). In addition, no ovarian tissue or interstitial gonads were found in MT-treated mandarin fish detected by all tissue sections.

The change in GSI of mandarin fish in control and MT treated groups were shown in Fig. 5. The results showed that GSI of the control and MT treated groups increased gradually from the 70 dpf to 240 dpf, and the highest value were at 240 dpf. GSI of MT treatments were significantly lower than that of the control group ($P < 0.05$) at 70dpf (termination of treatment), however, the value of the control group was significantly lower than that of the MT treatments at 120 dpf ($P < 0.05$). At 180 dpf and 240 dpf, there was no significant difference in GSI between the control and MT treated groups. During the experiment, the difference in GSI of mandarin fish in different concentrations of MT treatment group were not significant.

The changes of serum steroid hormone (T and E_2) in mandarin fish from 70 dpf to 240 dpf were shown in Fig. 6 and Fig.7. At termination of treatment (70dpf), the contents of T and E_2 of the control group were significantly higher than those in the MT treatment group ($P < 0.05$), and the difference in T and E_2 of mandarin fish in different concentrations of MT treatment group were not significant. When the 17 α -methyltestosterone treatment was completed, the levels of plasma T and E_2 of the MT-treated groups returned to normal, and there was no significant difference with the control group.

Illumina sequencing and unigenes annotation

Gonad cDNA libraries of nine testes and nine ovaries were sequenced. A total of 120,955,742 (97.72%) clean reads were obtained and used for de novo assembly. 60,971,396 and 59,984,346 clean reads were obtained from ovaries and testes, respectively, with a mean sequence length of 150bp (Table 3). After assembling and clustering, a total of 63,632 unigenes ranging from 201 to 21,004 bp with a mean sequence length of 1,182 bp were acquired (Fig. 8).

A total of 33,535 (52.7%) unigenes were annotated from the four databases, among which 33,471 unigenes were annotated from Nr database, 27,685 unigenes were annotated from Swissprot database, 22,501 unigenes were annotated from COG/KOG database and 20,323 unigenes were annotated from KEGG database (Fig. 9).

Analysis of differentially expressed genes

After RPKM standardized calculation, 46,391 DEGs were detected in the gonads of 120 dpf, among which 3,435 genes were highly expressed in ovaries and 42,956 in testes. When $RPKM \geq 2$, 811 and 1,530 genes were differentially expressed in ovaries and testes, respectively.

57,171 DEGs were assigned to three GO term categories including biological processes (28,349), cellular components (16,155), and molecular functions (12,667) (Fig. 10). In biological processes, the DEGs of the ovaries and testes were mainly concentrated in cellular process (647 and 5,290 DEGs in ovaries and testes, respectively), metabolic process (547 and 3,850 DEGs in ovaries and testes, respectively) and single-organism process (532 and 4,719 DEGs in ovaries and testes, respectively). In cell components, the DEGs of the ovaries and testes were mainly concentrated in the cell (431 and 2,806 DEGs in ovaries and testes, respectively), cell part (431 and 2806 DEGs in ovaries and testes, respectively), membrane (256 and 2,610 DEGs in ovaries and testes, respectively), membrane part (218 and 2356 DEGs in ovaries and testes, respectively) and organelle (307 and 1,905 DEGs in ovaries and testes, respectively). In molecular functions, the DEGs of the ovaries and testes were mainly concentrated in binding (606 and 4,865 DEGs in ovaries and testes, respectively) and catalytic activity (423 and 3,352 DEGs in ovaries and testes, respectively).

According to KEGG classification of DEGs, 5,378 DEGs were mapped to 260 different pathways. Among these pathways, 36 pathways were the prominent pathways ($P \leq 0.05$) which mainly consisted of Neuroactive ligand-receptor interaction (443 Unigene, 8.24%), Calcium signaling pathway (409 Unigene, 7.61%), and Cell adhesion molecules (CAMs) (250 Unigene, 4.65%). Moreover, MAPK signaling pathway (418 Unigene, 7.77%) and Steroid hormone biosynthesis (42 Unigene, 0.78%) were the pathways related to reproductive development (Table 4).

Identification of DEGs associated with sex development

By analyzing the transcriptome data of ovaries and testes, the expression levels of 7 genes related to sex determination and differentiation were listed in Table 5. We noticed that *dmrt1*, *amh*, *sox9*, *gsdf* and *dax1* expression in the testes were higher than ovaries ($P < 0.05$), while *foxl2* and *cyp19a1a* expression in the ovaries were higher than testes ($P < 0.05$).

Real-time PCR verifications

At termination of treatment (70dpf), the relative expression of *dmrt1*, *sox9*, *foxl2* and *cyp19a1a* in the gonad were shown in fig.11 and fig.12. The relative expression of *dmrt1*, *sox9* in M0 (♂), M1 and M2 were significantly higher than that in M0 (♀), but the relative expression of *foxl2* and *cyp19a1a* were significantly lower than that in M0 (♀) ($p < 0.05$). In different concentrations of MT treatment groups and M0 (♂), the relative expression of *dmrt1*, *sox9*, *foxl2* and *cyp19a1a* were not significantly different ($p > 0.05$).

Discussion

The anabolic effects of MT on growth have been reported in some teleost fish, but the effect of MT on growth was not the same. Some studies suggest that low concentration of MT can promote the growth of fish, such as Eurasian perch (*Perca fluviatilis*) [25], common carp (*Cyprinus carpio* L.) [26], brook trout (*Salvelinus fontinalis*) [27] and zebrafish [28]. This may be due to the role of androgens in stimulating appetite and accelerating protein synthesis in fish [29]. Moreover, some research held that high concentration of MT inhibited fish growth, such as black molly (*Poecilia sphepops*) [30], sablefish (*Anoplopoma fimbria*) [31], brook trout [11] and Atlantic cod (*Gadus morhua*) [32]. The results of this study showed that different concentrations of MT induced marked inhibition on the growth of treatment groups, indicating that the MT concentration we selected may have exceeded the upper limit of promoting fish growth, and the treatment concentration was too high to promote the growth of mandarin fish. After hormonal treatment, growth effects as compensatory growth

effects may occur in some fish [33, 34]. The same phenomenon was found in our study which indicated that this may be a sign of compensatory growth. The treatment intensity of MT was related to the treatment time and concentration. When the treatment intensity of MT was low, it had no significant effect on the survival rate of the treatment group. Nevertheless, when the intensity of MT treatment was too high, the survival rate of the treatment group was significantly lower than that of the control group [35]. Research found that zebra cichlid (*Cichlasoma nigrofasciatum*) was fed with high-dose estradiol a few days, the mortality of treatment group was significantly higher than that of the control group [36]. Furthermore, yellow catfish was induced with low-dose 17β -estradiol and 17α -methyltestosterone for a long time, and the results showed that the survival rate of the treatment group was significantly lower than that of the control group [30]. However, there was no significant difference in mortality between the treatment group and the control group when the European sea bass (*Dicentrarchus labrax* L.) [37] was fed with low-dose MT for a short time. In this study, we found that mandarin fish treated with dietary MT at 50 mg/kg and 100 mg/kg dosages for 2 months, the survival rate was slightly lower than that of the control group, which indicated that the concentration of MT can be used to induce the sex reversal mandarin fish, and the treatment intensity was more appropriate.

Appropriate concentration of MT treatment can promote the gonadal development and make the gonads present large volumes of spermatid in advance [28, 35], which were consistent with the result of our research. It was found that MT treatment may lead to gonadal atrophy, hermaphrodites and female ratio increased [38–40]. For example, the GSI of the treatment group was lower than that of the control group when Nile tilapia was treated with MT at a concentration of 40 mg/kg [41]. Likewise, Phelps, et al. also found that GSI of the treatment group was lower than that of the control group in Nile tilapia [7]. In Atlantic cod, MT was successful in producing hermaphrodites (46.7%) in the groups that were treated for the longest time period at the high dose [34], and hermaphrodites also were found in largemouth bass (*Micropterus salmoides*) [42]. Davis, et al. [43] reported that 60 mg / kg MT added to feed the channel catfish (*Ictalurus punctatus*), resulting in 99% of females in the treatment group. In this study, no androgynous phenomenon was found and sex ratios were 100% males at the end of MT treatment. However, gonadal atrophy occurred in both MT-treated groups and MT-treated testes were significantly inhibited at the end of hormone induction. With the end of MT treatment, the GSI of the MT treatment group gradually did not differ from the control group. This may be due to positive correlation between the GSI and sex steroids testosterone (T) [44, 45]. MT destroyed the balance of hormone levels in mandarin fish and then the content of T in the serum decreased significantly, which affected the GSI of the MT-treated groups. While once the MT treatment finished, the T content in the serum returned to normal, and the GSI of MT-treated groups also returned to normal.

Sex steroids testosterone (T) and 17β -estradiol (E_2) play important roles in gonadal differentiation and gonadal development in fish [46–49]. MT is a synthetic testosterone analogue that is absorbed by fish and affects the balance of hormone levels by affecting the expression levels of steroidogenic synthase genes and their regulatory factors [50, 51]. Studies have shown that amh can mediate the down regulation of testosterone biosynthesis, and androgen treatment can lead to the increase relative expression of amh [52–54]. The results of our research showed that MT treatment inhibited the levels of serum steroid hormone (T and E_2), which indicated that MT may break the original balance of hormone levels in the fish by affecting the expression of genes related to the synthesis of hormone.

In this study, we investigated the effects of MT treatment on mandarin fish gonadal transcriptomes during gonadal differentiation and screened out the genes related to the sex determination and differentiation. MT activated gonadal expression of pro-male genes *dmrt1* and *sox9* and repressed gonadal expression of pro-female genes *cyp19a1a* and *foxl2*. We found strong upregulation of *dmrt1* expression in control group in males and MT-treated testes in this study, consistent with the research of honeycomb grouper (*Epinephelus merra*) [55]. In zebrafish, upregulation of *dmrt1* expression is associated with androgen-induced gonadal masculinization [6]. Similarly, the expression of *dmrt1* was found upregulation in sex reversal of rainbow trout [56] and medaka [57]. It is indicated that the *dmrt1* may be a key gene in the gonadal masculinisation in fish. MT treatment increased *sox9* expression in mandarin fish gonads. *Sox9* is an important gene associated with male sex differentiation and gonad development [58], and there are two *sox9* (*sox9a* and *sox9b*) genes in most teleost [59]. The expression of *sox9* in the testis, ovary, brain, liver and heart of rainbow trout were detected and the results showed that the gene was only significantly expressed in the testis and brain [60]. Similar to rainbow trout, *sox9b* were found to be expressed at higher levels in the testes than ovaries in Nile tilapia [61, 62]. In orange-spotted grouper, the expression level of *sox9* upregulated significantly and the results of tissue section showed that the gonad of it changed from ovary to testis during MT induction, indicating that *sox9* may be an important factor in triggering and maintaining masculinity of orange-spotted grouper [63]. Our results suggest that *sox9* may play an important role in the differentiation and development of testis. *Foxl2* belongs to the forkhead transcription factors family and plays an important role in the development and maintenance of ovarian function in vertebrate animals [64]. In fish such as rainbow trout [65], Japanese flounder (*Paralichthys olivaceus*) [66] and Nile tilapia [67], the expression level of *foxl2* in ovary was significantly higher than that in testis. In addition, in female Nile tilapia who knocked out *foxl2*, the E_2 levels and *cyp19a1a* expression levels in gonad decreased, resulting in their gonadal masculinization [68]. In our study, the expression level of *foxl2* in the ovary of mandarin fish was significantly higher than that in

the testis, indicating that *foxl2* is a key factor in the development of ovary. Our study showed that the expression of *cyp19a1a* in the ovary of mandarin fish was significantly higher than that in the testis. Aromatase cytochrome P450 encoded by *cyp19a1a* is a key enzyme in estrogen synthesis [69, 70]. In Nile tilapia [71], European sea bass [72] and Japanese eel (*Anguilla japonica*) [73], the expression of *cyp19a1a* was markedly higher in the ovary than that in testis. During the sexual differentiation of rainbow trout, *cyp19a1a* was highly expressed in ovaries [74]. We suggest that *cyp19a1a* may be one of the important gene in the development of the ovary. In a sense, the male-restricted expression of *dmrt1*, *sox9* and no female-specific expression of *cyp19a1a*, *foxl2* further confirmed the masculinization of mandarin fish by MT at the molecular level.

Conclusions

MT works well to produce all-male stock of mandarin fish. The growth of MT treatment and control groups had no significant difference from 180 dph to 240 dph, but the development of testis was promoted by MT. This work screened out the genes related to the sex determination and differentiation of the fish by sequencing and analysis of the transcriptome of the ovary and testis. Characterization and expression profiles of sex-determining genes were examined during gonadal differentiation by MT induction which further confirmed our results that MT can successfully induce sex reversal mandarin fish. Future studies will use sex reversal fish to crossed with selectively bred females to set up different families for progeny tests. Further selection of these brooders will be conducted based on whether they can produce all-female progeny.

Methods

Ethics statement

Mandarin fish experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and performed in accordance with the guidelines for experimental animals established by this committee.

Hormone treatment

Mature males (n=10) and females (n=10) of mandarin fish were collected from aquatic breeding farm in Foshan, Guangdong Province in April. After the injection of oxytocin, the fish were maintained in ambient water temperature of 25°C-27°C. After the broodstock estrus, the larvae were obtained by artificial insemination, then incubated at water temperature of 25°C -28°C.

The androgen used in this experiment was 17 α -methyltestosterone, and the concentration of it were 50 mg/kg and 100 mg/kg. At 10 dpf, undifferentiated juvenile mandarin fish (n=4500) were randomly selected to conduct induction experiment of sex reversal for 2 months. The experiment was divided into three groups: M0 (control group), M1 (50 mg/kg treatment group), M2 (100 mg/kg treatment group), and set up three parallels in each group. Each parallel consists of 500 individuals which were respectively maintained in 4 × 4 m tanks at water temperature of 25-28°C and natural photoperiod.

Evaluation of growth status and sex ratios

During hormone induction, the 10 dpf juvenile mandarin fish were taken every 10 days and 30 samples of each replicate were taken to measure the body weight (accurate to 0.001g) and body length (accurate to 0.01cm). At the termination of treatment, the survival rate was counted and each parallel including 30 individuals were dissected to obtain external morphology of the gonads which used to determine the gonadal sex of each fish and sex ratios for each exposure group.

At the termination of hormone induction, 300 individuals were randomly selected from the control group and MT treatment groups respectively, and then transferred into 20×20 m cement pool at water temperature of 25-30°C with natural photoperiod. Each group consisted of 3 biological replicates comprising 900 juvenile fish each. At 120 dpf, 180 dpf, and 240 dpf, 30 individuals of the control group, M1 treatment group, and M2 treatment group were randomly selected from the cement pool, which were anesthetized by MS-222 to measure body weight and body length.

Determination of gonadal morphology and serum steroid hormone

At 70dpf (termination of treatment), 120 dpf, 180 dpf, and 240 dpf, 30 individuals were randomly selected from the control group (male gonads), M1 treatment group, and M2 treatment group respectively, which were anesthetized by MS-222 and dissected to obtain the

individual gonads. After weighed body weight and gonad weight, gonads were transferred to Bouin's solution, and then gonadosomatic index (GSI) were calculated using the formula: $GSI = (\text{gonad weight/body weight}) \times 100$. The gonad tissues were fixed overnight in Bouin's solution, dehydrated and embedded in paraffin. The samples were serially sliced by HM340 paraffin slicer to a thickness of 6-8 μm . Haematoxylin and eosin-stained sections were examined under light microscopy to determine gonadal morphology.

At 70dpf, 120 dpf, 180 dpf, and 240 dpf, 9 individuals were randomly selected from the control group, M1 treatment group and M2 treatment group respectively, and blood samples were taken from the caudal vessel with a heparinized needle and syringe. The samples were allowed to kept on crushed ice until centrifuged at 8000 rpm for 15 min, and the supernatant was stored at -80°C. The sex steroids testosterone (T) was measured by the testosterone radioimmunoassay kit (Cat. No. B10PZA, Beijing North Biotechnology Institute, Beijing, China) [23] and 17 β -estradiol (E₂) was analyzed by enzyme-linked immunosorbent assay (ELISA) [24].

RNA extraction, complementary DNA library construction, and Illumina sequencing

The 4-month-old female (n=9) and male (n=9) mandarin fish used for transcriptome sequencing were collected from aquatic breeding farm in Foshan, Guangdong Province. The gonadal tissues were separated in 1.5 mL RNase-free centrifuge tubes, frozed in liquid nitrogen, and then transferred to -80 °C for storage.

Total RNA was extracted from each gonad using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and quality were determined using a NanoDrop spectrophotometer (Nano-Drop Technologies, Wilmington, DE, U.S.) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.). RNA integrity was assessed by ethidium bromide staining of the 28S and 18S ribosomal bands on a 1% agarose gel. Mixed the RNA of nine ovaries and testes with equal amounts, and built 2 RNA pools. Then sent them to Guangzhou Kiddio Biotech Co., Ltd. to build cDNA library. Libraries were sequenced using an Illumina HiSeq™ 4000 platform.

De novo assembly and functional annotation

Raw Data were cleaned by removal of adaptors, leading and trailing low quality or N bases (over the ratio of 10%), removal of bases when the average quality per base dropped below 20. Clean data were then de novo assembled using Trinity software (version 2.0.6).

To annotate all unigenes, BLASTx (version 2.2.26) with an E-value threshold of 1×10^{-5} was used to query the unigenes against different databases: NCBI nonredundant protein (Nr) database, Swiss-Prot protein database, Kyoto Encyclopedia of Genes and Genomes (KEGG) database and Clusters of Orthologous Groups (COG). Gene Ontology (GO) annotation and functional classification of unigenes were performed by using Blast2GO software.

Differential gene expression analysis

The reads per kilobase of exon region per million mappable reads (RPKM) method was used to quantify gene expression levels—the calculation formula

$$RPKM = \frac{10^6 C}{NL/10^3}$$

RPKM is the expression level of the gene, C is the only number of reads aligned to the gene, N is the total number of reads that are uniquely aligned to the reference gene, and L is the number of bases of the gene. The RPKM method can eliminate the effect of gene length and sequencing difference on gene expression. Therefore, the RPKM value can be directly used to compare gene expression differences between different samples.

FDR (False Discovery Rate) and log₂ (Ovary_RPKM/Testis_RPKM) were used to identify DEGs between ovaries and testes. FDR<0.05 and |log₂ (Ovary_RPKM/Testis_RPKM)| >1 between the testes and ovaries were considered as DEGs, which subjected to enrichment analysis by GO functional group and KEGG pathways.

Validation of differentially expressed genes using quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) was conducted on 4 differentially expressed transcripts identified from the RNA-seq data. Total RNA extraction was performed as mentioned above. The cDNA synthesis was performed using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, China). Specific primers designed for qPCR based on DEGs and internal reference gene 18S rRNA sequences. Real-time PCR was performed using the SYBR®Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, China), and the PCR reaction was performed using the LightCycler®480 System II Real-Time PCR System. Each qPCR reaction was performed in triplicate.

Statistical analysis

The results were expressed as mean $\pm$ SD. The percentage data were transformed by arcsin square root transformation. Data were subjected to one-way ANOVA followed by Duncan's multiple range test for post hoc comparisons of means. SPSS version 21.0 was used for the analyses in the study. The level of significance for all statistical tests was set at 5% ($p < 0.05$).

Abbreviations

T: testosterone; E2: 17 β -estradiol; LL: lobular lumen; MT: 17 α -methyltestosterone; PCR: polymerase chain reaction; DNA: deoxyribonucleic acid; RNA: ribonucleic acid; rpm: round per minute; RT-PCR: reverse transcriptase-polymerase chain reaction; RNA-seq: RNA sequencing; RPKM: Reads per kilobase per million mapped reads; SPC: spermatogenic cyst; SC: spermatocyte; SG: spermatogonia; SZ: spermatozoon; SM: spermatids; dph: days post-hatching

Declarations

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

SL, PX and GFL designed the experiments. SL and PX performed the treatments, sample collection and laboratory work with the assistance of YQS and HDY. SL and PX analysed and interpreted the RNASeq data with the assistance of XGL. XLC and DLG performed the mandarin fish histology. SL wrote the manuscript with contributions from SB and XPZ. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Mandarin fish experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and performed in accordance with the guidelines for experimental animals established by this committee.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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Tables

Table 1 The body weight and body length of *S. chautsi* during MT treatment

		M0	M1	M2
10	Weight(g)	0.0059±0.0027	0.0059±0.0027	0.0059±0.0027
	Length(cm)	0.79±0.10	0.79±0.10	0.79±0.10
20	Weight(g)	0.0857±0.0216*	0.0608±0.0151	0.0542±0.0130
	Length(cm)	1.59±0.15*	1.36±0.21	1.30±0.14
30	Weight(g)	0.6035±0.1717*	0.3341±0.1119	0.2744±0.0878
	Length(cm)	2.50±0.26*	2.14±0.24	2.01±0.27
40	Weight(g)	2.8117±0.9121*	1.4109±0.6381	1.2310±0.4246
	Length(cm)	3.60±0.35*	2.71±0.38	2.37±0.20
50	Weight(g)	5.6339±2.3485*	2.9122±1.0439	2.5070±1.0982
	Length(cm)	4.86±0.63*	4.21±0.71	4.16±0.28
60	Weight(g)	13.3250±5.0225*	8.4892±2.6375	6.4288±2.3676
	Length(cm)	7.47±0.66*	5.36±0.79	5.16±0.48
70	Weight(g)	20.2042±4.2142*	13.6494±2.9360	12.5314±3.9176
	Length(cm)	9.45±0.74*	6.83±0.92	6.54±0.54

Note: *Significant difference between control group and MT treatments ($P < 0.05$). Each experiment was repeated three times.

Table 2 Effects of MT on sex ratios in *S. chautsi*

Sample	Q1	Median	Q3	Q4	GC%
M0	90	46	44	51.11	
M1	90	90	0	100	
M2	90	90	0	100	

Table.3 Summary of *S. chuatsi* transcriptome

Sample	raw reads	clean reads (%)	GC%	Adapter (%)	low quality (%)
M0	60,863,104	59,984,346 (98.56%)	49.17%	31,418 (0.05%)	798,934 (1.31%)
M1	62,915,274	60,971,396 (96.91%)	51.27%	19,3200 (0.31%)	1,700,204 (2.7%)

Table.4 The pathway analysis of different expression genes

Pathway	Candidate genes with pathway annotation	All genes with pathway annotation	Pvalue	Pathway ID
Neuroactive ligand-receptor interaction	443 (8.24%)	550 (6.06%)	0.000000	ko04080
Calcium signaling pathway	409 (7.61%)	536 (5.91%)	0.000000	ko04020
Cell adhesion molecules (CAMs)	250 (4.65%)	316 (3.48%)	0.000000	ko04514
Cytokine-cytokine receptor interaction	238 (4.43%)	308 (3.4%)	0.000000	ko04060
Adrenergic signaling in cardiomyocytes	270 (5.02%)	374 (4.12%)	0.000000	ko04261
ECM-receptor interaction	158 (2.94%)	211 (2.33%)	0.000001	ko04512
Focal adhesion	350 (6.51%)	508 (5.6%)	0.000003	ko04510
MAPK signaling pathway	418 (7.77%)	616 (6.79%)	0.000003	ko04010
Vascular smooth muscle contraction	214 (3.98%)	299 (3.3%)	0.000005	ko04270
Hedgehog signaling pathway	87 (1.62%)	110 (1.21%)	0.000008	ko04340
Phosphatidylinositol signaling system	161 (2.99%)	223 (2.46%)	0.000033	ko04070
Gap junction	161 (2.99%)	225 (2.48%)	0.000072	ko04540
Linoleic acid metabolism	32 (0.6%)	37 (0.41%)	0.000326	ko00591
alpha-Linolenic acid metabolism	29 (0.54%)	33 (0.36%)	0.000351	ko00592
ABC transporters	83 (1.54%)	111 (1.22%)	0.000432	ko02010
AGE-RAGE signaling pathway in diabetic complications	175 (3.25%)	253 (2.79%)	0.000624	ko04933
Phototransduction	39 (0.73%)	48 (0.53%)	0.001015	ko04744
Cardiac muscle contraction	133 (2.47%)	191 (2.11%)	0.001799	ko04260
Steroid biosynthesis	29 (0.54%)	35 (0.39%)	0.002600	ko00100
Glycerophospholipid metabolism	120 (2.23%)	173 (1.91%)	0.003633	ko00564
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	32 (0.6%)	40 (0.44%)	0.004618	ko00532
Steroid hormone biosynthesis	42 (0.78%)	55 (0.61%)	0.005944	ko00140
Adipocytokine signaling pathway	95 (1.77%)	137 (1.51%)	0.009174	ko04920
Purine metabolism	234 (4.35%)	359 (3.96%)	0.011240	ko00230
Insulin signaling pathway	190 (3.53%)	289 (3.19%)	0.012929	ko04910
Insulin resistance	159 (2.96%)	241 (2.66%)	0.018178	ko04931
VEGF signaling pathway	105 (1.95%)	156 (1.72%)	0.023080	ko04370
Jak-STAT signaling pathway	140 (2.6%)	212 (2.34%)	0.024438	ko04630
beta-Alanine metabolism	36 (0.67%)	49 (0.54%)	0.027791	ko00410
Retinol metabolism	51 (0.95%)	72 (0.79%)	0.028179	ko00830
Inositol phosphate metabolism	112 (2.08%)	168 (1.85%)	0.028564	ko00562
Regulation of actin cytoskeleton	295 (5.49%)	464 (5.11%)	0.029177	ko04810
Melanogenesis	138 (2.57%)	210 (2.31%)	0.031378	ko04916
Nitrogen metabolism	21 (0.39%)	27 (0.3%)	0.035623	ko00910
Protein digestion and absorption	9 (0.17%)	10 (0.11%)	0.042096	ko04974
Ether lipid metabolism	54 (1%)	78 (0.86%)	0.044810	ko00565

Table.5 List of representative genes related to sex determination and differentiation in gonadal transcripts of *S.chuatsi*

GeneID	Gene Name	Annotation	Testis_RPKM	Ovary_RPKM	log2 Ratio (Ovary/Testis)	Pvalue	FDR
Unigene0000094	<i>dmrt1</i>	double sex and mab-3 related gene-1	65.2727	0.2708	-7.913108168	0	0
Unigene0041160	<i>amh</i>	Anti-Müllerian hormone	163.8657	2.7547	-5.894474883	0	0
Unigene0016462	<i>cyp19a1a</i>	cytochrome P450, family 19, subfamily A, polypeptide 1a	0.1008	2.0933	4.376211541	1.29676E- 29	4.55182E- 29
Unigene0004710	<i>sox9</i>	Sry-related HMG box-9	26.3341	0.3216	-6.355520938	0	0
Unigene0017031	<i>gsdf</i>	gonadal soma derived factor 1	2603.8135	51.4281	-5.661925453	0	0
Unigene0057507	<i>foxl2</i>	winged helix/forkhead transcription factor L2	1.7116	7.9548	2.216480075	3.43E-91	2.00E-90
Unigene0007455	<i>dax1</i>	Dosage-sensitive sex reversal Adrenal hypoplasia congenital critical region on the X chromosome gene	4.639	0.4789	-3.276017506	4.05E-54	1.84E-53

1

Figures

Figure 1

The body weight of *S. chautsi* post-treatment. Data presented are expressed as mean±SD (n=90). * indicate significant difference between control group and MT treatments (P<0.05). M0, control group; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 2

The body length of *S. chautsi* post-treatment. Data presented are expressed as mean±SD (n=90). * indicate significant difference between control group and MT treatments (P<0.05). M0, control group; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 3

Effects of MT on survival in *S. chautsi*. Data presented are expressed as mean±SD (n=3). M0, control group; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 4

Histological analysis of gonads of *S. chautsi* male in control and MT treated groups. a, Gonads of *S. chautsi* in control group at 70dph, showing spermatogenic cyst (SPC), spermatogonia (SG), spermatocyte(SC); b, Gonads of *S. chautsi* in 50 mg/kg MT treatment at 70dph, showing spermatogonia (SG), spermatocyte(SC); c, Gonads of *S. chautsi* in 100 mg/kg MT treatment at 70dph, showing spermatids (SM); d, Gonads of *S. chautsi* in control group at 120dph, showing spermatozoon (SZ); e, Gonads of *S. chautsi* in 50 mg/kg MT treatment at 120dph, showing spermatozoon (SZ) in lobular lumen(LL); f, Gonads of *S. chautsi* in 100 mg/kg MT treatment at 120dph, showing spermatozoon (SZ); g, Gonads of *S. chautsi* in control group at 180dph, showing spermatozoon (SZ) in lobular lumen(LL); h, Gonads of *S. chautsi* in 50mg/kg MT treatment at 180dph, showing spermatozoon (SZ); i, Gonads of *S. chautsi* in 100 mg/kg MT treatment at 180dph, showing spermatozoon (SZ); j, Gonads of *S. chautsi* in control group at 240dph; k, Gonads of *S. chautsi* in 50 mg/kg MT treatment at 240dph; l, Gonads of *S. chautsi* in 100 mg/kg

Figure 5

The gonadosomatic index (GSI) of *S. chausi* male in control and MT treated groups. Data presented are expressed as mean $\pm$ SD (n=30). * indicate significant difference between control group and MT treatments (P<0.05). M0, control group; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 6

Effects of 17 α -methyltestosterone on serum T levels in *S. chausi* male in control and MT treated groups. Data presented are expressed as mean $\pm$ SD (n=9). * indicate significant difference between control group and MT treatments (P<0.05). M0(♂), control group in males; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 7

Effects of 17 α -methyltestosterone on serum E2 levels in *S. chautsi* male in control and MT treated groups. Data presented are expressed as mean $\pm$ SD (n=9). * indicate significant difference between control group and MT treatments (P<0.05). M0(♂), control group in males; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 8

Length distribution of *S. chuatsi*-Unigene

Figure 9

Homologous genes in public databases of *S.chuatsi*-Unigene

Figure 11

Effects of 17 α -methyltestosterone on expression level of *dmrt1*, *sox9* in the gonads of *S. chausi* in control and MT treated groups. mRNA levels in the time-course were qualified by real-time RT-PCR and normalized by 18S rRNA. Data presented are expressed as mean $\pm$ SD (n=6). Different letters indicate significant difference between different groups (P<0.05). M0(♀), control group in females; M0(♂), control group in males; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

Figure 12

Effects of 17 α -methyltestosterone on expression level of *foxl2*, *cyp19a1a* in the gonads of *S. chausti* in control and MT treated groups. mRNA levels in the time-course were qualified by real-time RT-PCR and normalized by 18S rRNA. Data presented are expressed as mean $\pm$ SD (n=6). Different letters indicate significant difference between different groups (P<0.05). M0(♀), control group in females; M0(♂), control group in males; M1, 50 mg/kg MT treatments; M2, 100 mg/kg MT treatments

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