nature portfolio

Peer Review File

Steroid hormone-deprived sex reversal in cyp11a1 mutant XX tilapia experiences an ovary-like stage at molecular level

Corresponding Author: Professor Deshou Wang

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
OVERVIEW AND GENERAL RECOMMENDATION

The molecular mechanisms underlying sex differentiation in vertebrates, the exact role of sex steroid hormones and how mechanisms vary between organisms is yet to be fully understood and continue to be a significant area of research. This study by Xiao et al investigated the sex differentiaton and gonad development of sex steroid hormone-deficient male and female tilapia in comparison to wild type. CRISPR/Cas9 system was used to disrupt cyp11a1 gene which codes for an enzyme responsible for the biosynthesis of pregnenolone (P5), the precursor of all sex steroids hormones. Among the key findings include the progression of spermatogenesis in mutant males (although delayed compared to wild type) and the initial development of ovary-like stage in mutant females followed by sex reversal and (delayed) spermatogenesis. The sex reversal in mutant females was associated with trans-differentiation of gonadal cells instead of apoptosis. Finally, ovarian development in mutant females can be rescued by administration of P5 or E2 but not DHP.

The strength of this study is on the well-designed experiments that involved comprehensive analyses and techniques (qPCR, transcriptome, immunoassay, histology, in situ hybridization, immunofluorescence, etc.). Hence, the study is able to provide valuable insights that are based on robust data. The research subject is of great importance in molecular reproductive endocrinology (general, comparative, and applied), and fits well within the scope of this journal.

However, I have some questions/comments for the authors to address before I can recommend this manuscript for publication.

COMMENTS/QUESTIONS

1. Title. It would be relevant to indicate the model species in the title given that similar investigations have been done in mammalian models as well as in zebrafish. Also, why only say “estrogen-deprived” mutants when the trial was designed to target/remove all sex steroids and not just estrogens? Moreover, contrary to the title, only the cyp11a1 mutant females experienced an ovary-like stage but not the mutant males. Authors are encouraged to revise the title to better represent the study.

2. To investigate the role of sex steroids in fish gonad differentiation, this study disrupted the cyp11a1 gene to establish a line of tilapia that is free of all sex-steroids. However, in the results [L329-331], authors stated that “As expected, EIA measurement showed that the cyp11a1 KO fish displayed background serum P5, cortisol, E2, DHP, T, 11-KT level”. Why is this expected? This should be discussed in the Discussion section [L447-454] and backed with relevant references. Given that the mutant fish in this study had basal levels of steroids hormones, do they still qualify as steroid hormone-free model? Perhaps they are steroid hormone-deficient rather than steroid hormone-free.

3. Results [L335-336]. “Mutation of cyp11a1 results in female to male sex reversal and defects in spermatogenesis” [also in Figure 2, L851; Discussion L455]. The term “female to male sex reversal” only applies to mutant females but not in mutant males, since results show that mutant males displayed testicular differentiation without going through an ovary-like stage hence it could not be called female to male sex reversal. Authors are encoured to revise the result subtitle [L335-336], discussion subtitle [L455], and figure 2 title [L851].

4. Results. Histological analysis (Fig. 2) showed that testis of mutant males and females contained spermatogonia and spermatocytes hence indicating the progression of spermatogenesis, albeit slower than wild type males. Should this be

referred to as “defective”? In my opinion, results indicate a “delayed spermatogenesis” rather than a “defective spermatogenesis”. I suppose whether spermatogenesis is defective or not ultimately depends on the production of spermatozoa that is capable of fertilization (i.e., through fertilization trial). Or perhaps presence of abnormal histological features in testis (e.g., disorganized tubular structure). However, such information is lacking. Whether the spermatozoa produced by mutant males and females are defective or not can be subject of future studies.

5. Results. [Fig. 4] Why is gene expression data and IF result for WTXX not included? For gene expression of markers associated with various stages of spermatogenesis, authors pointed out that values are lower in KOXX and KOXY compared to WTXY. However, these genetic markers are expressed, nonetheless. Expression is likely higher compared to that of WTXX and enough to support spermatogenesis (albeit delayed) as shown in histological analysis, and as pointed out in the previous question.

6. Results. Please include gonad histological image of control KOXX during rescue trial in Fig. 7. Also briefly describe the control in results section [L437-445].

Reviewer #2

(Remarks to the Author)
This manuscript, entitled “estrogen-deprived sex reversal in cyp11a1 mutants experiences an ovary like stage at molecular level” by Xiao and colleagues show the importance of steroid synthesis for ovary development in a teleost species, the Nile Tilapia. The question raised by the authors is definitely worth asking and they provide a large range of methodologies and approaches to provide some answers. The article is clear and numerous results are provided. I have a few comments that need to be addressed, notably regarding the conclusions.
In general, I find the description of the methods and the details provided by the authors a little bit to short, especially regarding the image analysis:
- How was selected the zone(s) to be analyzed, Image J was used but what types of parameters and how were they defined?
- 4 populations of tilapia where analyzed for Mendelian distribution, how many fish per population and how were obtained this populations?
- unless I misunderstood, all transcriptomic analysis was performed with n=1 per group, making this data impossible to interpret (p values are mentioned to be <0.05 in material and methods, how is this possible?). Even if authors used a pool of 3, there is still one sample analysed. Figures 2R and S, figure 4 G and figures 6 H, K and L are really nice but unfortunately, we cannot not rely on the results from n=1 (again unless I misunderstood the material and methods).
Lines 345-346: Cyp 11c is expressed in cyp11a1KO XX fish as shown in figure 2 T and 2 N
Lines 348-350 and figure 2Q: I do not understand what was correlated, what is the global gene expression pattern and how was it calculated?
Line 363: and Figure 3: Fish were treated with androgen, which one and how? There is no statistics, number of animals… In addition, and this is the reason for statistics, the image presented figure 3 highlights a very strong expression of aromatase in KOXX, much higher than WTXX, and definitely much higher compared to what is shown for these 2 genotypes in figure 2.
Lines 363-367 and discussion line 498-502. If I understand correctly, KT (androgen) in vitro is able to inhibit sf-1 dependent aromatase expression via a (direct) action on the promoter. How come that cyp11a1KO male, with no androgen, do not show upregulation of aromatase? In addition, the conclusion that androgens are not important to induce male sexual differentiation does not fully hold with this in vivo model as there is no androgen in addition to no estrogen. Furthermore, the invitro model confirm that KT, via the activation of AR2, blocks the aromatase promoter, strongly suggesting the importance of androgens to further inhibit aromatase synthesis and therefore to reduce even more estrogen synthesis.
Line 527: Even if I stand on my previous comment on the issue with transcriptomic interpretation (n=1), the simple reading of the number of DEG in KO XX versus WT KO is definitely not highly similar (over 2700 DEG)
Line 534: In line with the previous comment, the current data does not demonstrate the trans-differentiation of germ cells from females to males. I agree that female gonads from KO XX show DMRT1 expression, but this expression happens in both germ and somatic lines. Furthermore, both foxl3 and DMRT1 are expressed in the female KO. The quality of the images figures 6 V to A’ does not allow to define whether there is colocalization within the same cells. Authors should also provide information of KO XY. More specific marker for male or female germinal cells should be used.
The steroid analysis in figure 1 is very interesting and this suggest that there still is some steroids to be produced, while there should not be anything left. Any interpretation, hypothesis to explain this presence while the cyp11a KO should be completely devoid of steroid?

Altogether, this is an interesting model that needs further study and should help in the understanding of steroid impact on sexual differentiation in Tilapia. I would also briefly discuss the potential impact of temperature on this model and the cortisol (or lack of) on ovary development and maintenance.

Author Rebuttal letter:

Dear Reviewers,
Thank you very much for your comments concerning our manuscript entitled “Estrogen-deprived sex reversal in cyp11a1 mutants experiences an ovary-like stage at molecular level”. Those comments are all valuable and very helpful for revising and improving our manuscript. We have studied the comments carefully and tried our

best to revise our manuscript. All the revisions have been marked in blue in our revised manuscript. It should be noted that in our revised manuscript, we use “cyp11a1-/-” instead of “cyp11a1 KO” to represent cyp11a1 homozygous mutants. We would like to show the details as follows:

Reviewer: #1
The molecular mechanisms underlying sex differentiation in vertebrates, the exact role of sex steroid hormones and how mechanisms vary between organisms is yet to be fully understood and continue to be a significant area of research. This study by Xiao et al investigated the sex differentiation and gonad development of sex steroid hormone-deficient male and female tilapia in comparison to wild type. CRISPR/Cas9 system was used to disrupt cyp11a1 gene which codes for an enzyme responsible for the biosynthesis of pregnenolone (P5), the precursor of all sex steroids hormones. Among the key findings include the progression of spermatogenesis in mutant males (although delayed compared to wild type) and the initial development of ovary-like stage in mutant females followed by sex reversal and (delayed) spermatogenesis. The sex reversal in mutant females was associated with trans-differentiation of gonadal cells instead of apoptosis. Finally, ovarian development in mutant females can be rescued by treatment of P5 or E2 but not DHP. The strength of this study is on the well-designed experiments that involved comprehensive analyses and techniques (qPCR, transcriptome, immunoassay, histology, in situ hybridization, immunofluorescence, etc.). Hence, the study is able to provide valuable insights that are based on robust data. The research subject is of great importance in molecular reproductive endocrinology (general, comparative, and applied), and fits well within the scope of this journal.
However, I have some questions/comments for the authors to address before I can recommend this manuscript for publication.
1. Title. It would be relevant to indicate the model species in the title given that similar investigations have been done in mammalian models as well as in zebrafish. Also, why only say “estrogen-deprived” mutants when the trial was designed to target/remove all sex steroids and not just estrogens? Moreover, contrary to the title, only the cyp11a1 mutant females experienced an ovary-like stage but not the mutant males. Authors are encouraged to revise the title to better represent the study.
Reply: Thanks for the reviewer’s suggestion. We have revised the title of our manuscript as “Steroid hormone-deprived sex reversal in cyp11a1 mutant XX tilapia experiences an ovary-like stage at molecular level”. Accordingly, we modified the text of our manuscript, especially in the Discussion section, to make it more relevant to the title(Line 548-549, 576-583).
2. To investigate the role of sex steroids in fish gonad differentiation, this study disrupted the cyp11a1 gene to establish a line of tilapia that is free of all sex-steroids. However, in the results [L329-331], authors stated that “As expected, EIA measurement showed that the cyp11a1 KO fish displayed background serum P5, cortisol, E2, DHP, T, 11-KT level”. Why is this expected? This should be discussed in the Discussion section [L447-454] and backed with relevant references. Given that the mutant fish in this study had basal levels of steroids hormones, do they still qualify as steroid hormone-free model? Perhaps they are steroid hormone-deficient rather than steroid hormone-free.
Reply: cyp11a1 is the only gene currently known to catalyze the production of P5, the precursor of all steroid hormones. In tilapia, only one cyp11a1 gene exists (Parajes et al., 2013), and therefore, we have undoubtedly created a steroid hormone-free model by mutation of cyp11a1 in this study. The basal levels of steroids hormones detected in cyp11a1 mutants (Figure 1) is more likely due to the non-specific binding of the first and second antibody of the EIA kit. We have discussed it in the Discussion section of our revised manuscript (Line 481-486). To avoid misunderstanding, we have modified the Abstract (Line 26) and Results section (Line 357-359) in our revised manuscript.
3. Results [L335-336]. “Mutation of cyp11a1 results in female to male sex reversal and defects in spermatogenesis” [also in Figure 2, L851; Discussion L455]. The term “female to male sex reversal” only applies to mutant females but not in mutant males, since results show that mutant males displayed testicular differentiation without going through an ovary-like stage hence it could not be called female to male sex reversal. Authors are encouraed to revise the result subtitle [L335-336], discussion subtitle [L455], and figure 2 title [L851].
Reply: Thanks for the reviewer’s suggestion. We have revised the Result subtitle, Discussion subtitle and Figure 2 title in our revised manuscript. The Result subtitle has been revised as “Mutation of cyp11a1 results in female to male sex reversal of XX fish and defects in spermatogenesis” (Line 363-364). The Discussion subtitle has been revised as “Steroid hormone-deprivation results in female to male sex reversal of XX

fish" (Line 493). The Figure 2 title has been revised as "Female to male sex reversal of XX fish after cyp11a1 mutation" (Line 906).
4. Results. Histological analysis (Fig. 2) showed that testis of mutant males and females contained spermatogonia and spermatocytes hence indicating the progression of spermatogenesis, albeit slower than wild type males. Should this be referred to as "defective"? In my opinion, results indicate a "delayed spermatogenesis" rather than a "defective spermatogenesis". I suppose whether spermatogenesis is defective or not ultimately depends on the production of spermatozoa that is capable of fertilization (i.e., through fertilization trial). Or perhaps presence of abnormal histological features in testis (e.g., disorganized tubular structure). However, such information is lacking. Whether the spermatozoa produced by mutant males and females are defective or not can be subject of future studies.
Reply: Thanks the reviewer for pointing this out. We stated that the spermatogenesis in mutant males was defective because the spermatogenesis could not continue and no fertile sperm was produced in adult mutant fish. To support our point, we have provided new data about the spermatogenesis of mutant males at 180 days after hatching in Figure 4 in our revised manuscript, and modified the Material and Methods section (Line 180-181, 189-204) and Results section (Line 404-411) accordingly.
5. Results. [Fig. 4] Why is gene expression data and IF result for WTXX not included? For gene expression of markers associated with various stages of spermatogenesis, authors pointed out that values are lower in KOXX and KOXY compared to WTXY. However, these genetic markers are expressed, nonetheless. Expression is likely higher compared to that of WTXX and enough to support spermatogenesis (albeit delayed) as shown in histological analysis, and as pointed out in the previous question.
Reply: As shown in the reply to the previous question, we still think the spermatogenesis in mutant males is defective, since the spermatogenesis did not restore and no fertile sperm was produced in adult mutant fish. We have provided new data in Figure 4 of our revised manuscript to support the defective spermatogenesis in cyp11a1 mutants. We believe that the point raised by the reviewer to show the expression of markers genes in the gonads of WT XX fish in Figure 4 is reasonable. However, in Figure 2, we have shown the female to male sex reversal of cyp11a1 mutant XX fish. In Figure 4, we would like to show the data that is closely related to the spermatogenesis of cyp11a1 mutant males (including sex reversed mutant XX fish and mutant XY fish). We are afraid that including the expression of marker genes in the gonads of WT XX fish in Figure 4 might interrupt the rationale of the manuscript. For your consideration, we have put the gene expression data for WT XX below.

6. Results. Please include gonad histological image of control KOXX during rescue trial in Fig. 7. Also briefly describe the control in results section [L437-445].
Reply: Thanks for the reviewer's suggestion. We have included histological images of WT XX and cyp11a1 mutant XX fish in Figure 7 of our revised manuscript, and modified the Figure legend and Results section (Line 476-479) accordingly.

Reviewer #2:
This manuscript, entitled "estrogen-deprived sex reversal in cyp11a1 mutants experiences an ovary like stage at molecular level" by Xiao and colleagues show the importance of steroid synthesis for ovary development in a teleost species, the Nile Tilapia. The question raised by the authors is definitely worth asking and they provide a large range of methodologies and approaches to provide some answers. The article is clear and numerous results are provided. I have a few comments that need to be addressed, notably regarding the conclusions.
1. In general, I find the description of the methods and the details provided by the authors a little bit too short, especially regarding the image analysis: -How was selected the zone(s) to be analyzed, Image J was used but what types of parameters and how were they defined? -4 populations of tilapia where analyzed for Mendelian distribution, how many fish per population and how were obtained this populations?
Reply: Sorry for the missing information. The zones selected for quantification of gsdf, Cyp19a1a and TUNEL positive signals in Figure 5 and Figure 6 were in the middle of the early gonads as shown in the figures. The zones selected for quantification of Cyp19a1a positive signals in gonads at 90 dah in Figure 3 were the whole gonads. The positive signal in all figures was quantified by Image J Pro 1.51 software using default parameters. We have provided more details about image analysis in the Materials and Methods section of our revised manuscript (Line

253-255, 317-319).
For Mendelian distribution analysis in the F2 populations at 5, 15, 25, 35 and 90 dah, four populations were calculated at each time point. For each population, more than 48 fish were randomly selected and analyzed. We have provided detailed information about the number of fish calculated in each population in Table S2 and modified the Materials and Methods section (Line 125-127) and Figure legend of Figure S2 (Line 1065-1067) in our revised manuscript.
2. Unless I misunderstood, all transcriptomic analysis was performed with n=1 per group, making this data impossible to interpret (p values are mentioned to be <0.05 in material and methods, how is this possible?). Even if authors used a pool of 3, there is still one sample analyzed. Figures 2R and S, figure 4 G and figures 6 H, K and L are really nice but unfortunately, we cannot rely on the results from n=1 (again unless I misunderstood the material and methods).
Reply: It is undeniable that transcriptome analysis with one sample will weaken the reliability of the results in this study. However, our conclusions were not solely based on the results of transcriptome analysis in this study. In Figure 2, we intended to present two findings: the sex reversal of cyp11a1 mutant XX fish and the continuous expression of cyp19a1a in the gonads of sex reversed mutant XX fish. These two findings were supported by several results, including results of histological analysis, transcriptome analysis, IF and qPCR. In Figure 4, we would like to show the defective spermatogenesis of cyp11a1 mutant fish by the results of IF and transcriptome analysis. We have also provided new data in Figure 4 to support the defective spermatogenesis of the mutant fish in our revised manuscript. All data including the results of IF, transcriptome analysis, histological analysis, Pap staining, motility test and fertilization assay in Figure 4 proved the defective spermatogenesis of the mutant fish. In Figure 6, we used the transcriptome data to confirm the ovary developing stage of the gonads in cyp11a1 mutant XX fish captured by FISH and to assess the apoptotic feature of the germ cells during ovary to testis transition. On one hand, we agree that it will make sense if we can provide transcriptome data with at least three biological replicates. However, it is almost impossible to finish this work due to the decreased fertility of the female heterozygous mutants and the difficulty in gonads collection at 15 days after hatching for four populations of fish. On the other hand, we think the results presented in Figure 6 including FISH, transcriptome analysis and TUNEL assay well supported the conclusion that the germ cells in the gonads of the mutant XX fish undergo transdifferentiation instead of apoptosis.
The differential gene expression analysis in this study was performed using edgeR software (Robinson et al., 2010). The p values was calculated according to the suggested BCV (square-root-dispersion) value (BCF=0.1). In the first version of our manuscript, we provided wrong information about this. We have revised the differential gene expression analysis in the Materials and Methods section of our revised manuscript (Line 298-302).
3. Lines 345-346: Cyp11c1 is expressed in cyp11a1 KO XX fish as shown in figure 2 T and 2 N
Reply: Thank you for pointing out this. We have revised the description about Figure 2 T and 2 N in the Results section (line 371-372) of our revised manuscript.
4. Lines 348-350 and figure 2Q: I do not understand what was correlated, what is the global gene expression pattern and how was it calculated?
Reply: Figure 2Q is the result of Pearson correlation analysis of gene expression in the gonads of WT XX, cyp11a1-/- XX, cyp11a1-/- XY and WT XY fish at 90 dah. It was calculated by sample relationship analysis tools on Omicshare web server (https://www.omicshare.com) based on the global gene expression of each sample. The number inside the square is the Pearson correlation coefficient between two samples. The closer the correlation coefficient gets to 1, the higher the similarity of global gene expression patterns between samples. To make this result more readable, we modified the Materials and Methods section (Line 294-298), Results section (Line 374-375) and Figure legends of Figure 2Q (Line 914-918) in our revised manuscript.
5. Line 363: and Figure 3: Fish were treated with androgen, which one and how? There is no statistics, number of animals… In addition, and this is the reason for statistics, the image presented figure 3 highlights a very strong expression of aromatase in KOXX, much higher than WTXX, and definitely much higher compared to what is shown for these 2 genotypes in figure 2.
Reply: Sorry for the missing information. Androgen treatment of the mutant XX fish in this study was conducted from 75 to 90 dah with a diet containing 11-KT at a dosage of 50 μg/g. After treatment, the expression of Cyp19a1a in the gonads was evaluated by IF (n=4/genotype). We have added these information in the Materials and Methods section (Line 315-319), provided statistical analysis in Figure 3 and modified the Figure legend accordingly (Line 939-941) in our revised manuscript. In order to keep the consistency of the results shown in Figure 2 and Figure 3 and avoid misunderstanding, we replaced the image of mutant XX fish in Figure 3 of our revised

manuscript.
6. Lines 363-367 and discussion line 498-502. If I understand correctly, KT (androgen) in vitro is able to inhibit sf-1 dependent aromatase expression via a (direct) action on the promoter. How come that cyp11a1 KO male, with no androgen, do not show up-regulation of aromatase? In addition, the conclusion that androgens are not important to induce male sexual differentiation does not fully hold with this in vivo model as there is no androgen in addition to no estrogen. Furthermore, the in vitro model confirm that KT, via the activation of AR2, blocks the aromatase promoter, strongly suggesting the importance of androgens to further inhibit aromatase synthesis and therefore to reduce even more estrogen synthesis.
Reply: From our understanding, we think that androgen deficiency is only the reason for the continuous expression of cyp19a1a in the gonads of the sex reversed cyp11a1 mutant XX fish. In the gonads of the genetic male fish (XY fish), the expression of cyp19a1a was inhibited by many male pathway genes other than androgen synthetase, such as dmrt1 and amhy as we reported previously (Wang et al., 2010; Liu et al., 2021). Therefore, even though cyp11a1 mutant XY fish were unable to synthesize androgens, the expression of Cyp19a1a in gonads was not up-regulated. In the Discussion section, we would like to declare that it is estrogen, not androgen, that determines the sex of fish, the androgen-induced sex reversal can be mediated by inhibition of Sf1 activated cyp19a1a transcription. The statement that androgen is not involved in fish sex determination is based on the following facts: 1. No steroidogenic/androgenic enzyme genes were found to be expressed in the early gonads of XY fish in tilapia by IHC, qPCR and transcriptomic analyses (Ijiri et al., 2008; Tao et al., 2013, Zheng et al., 2020). 2. No sex reversal has been reported when androgen synthetase or androgen receptor is disrupted in tilapia and other fish species (Crowder et al., 2018; Tang et al., 2018; Yu et al., 2018; Alward et al., 2020; Oakes et al., 2020; Zhang et al., 2020; Zheng et al., 2020; Ogino et al., 2023). 3. In this study, the sex differentiation of the cyp11a1 mutant XY fish was not affected and the gonads of cyp11a1 mutant XX fish could still reversed to testes even in the absence of androgen. We have modified the Discussion in our revised manuscript to make our point more clear (Line 517-531, 545-547).
7. Line 527: Even if I stand on my previous comment on the issue with transcriptomic interpretation (n=1), the simple reading of the number of DEG in KO XX versus WT KO is definitely not highly similar (over 2700 DEG).
Reply: From the results presented in Figure 2 including histological analysis, transcriptome analysis, IF and qPCR, we believe that the cyp11a1 mutant XX fish experienced female to male sex reversal, and the global gene expression pattern in the gonads of XX mutants was similar to that of WT XY fish at 90 dah. Given that there are still more than 2700 DEGs in the gonads of XX mutants and WT XY fish as the reviewer mentioned, we modified the description in the Discussion section of our revised manuscript as “Transcriptome analysis showed that at 15 dah, the global gene expression pattern in the gonads of the mutant XX fish was relatively more similar to those of the WT XX fish, but at 90 dah, it was relatively more similar to those of the WT XY fish” (Line 570-573). The defective spermatogenesis of the mutant male fish can also contribute to the identified DEGs.
8. Line 534: In line with the previous comment, the current data does not demonstrate the trans-differentiation of germ cells from females to males. I agree that female gonads from KO XX show DMRT1 expression, but this expression happens in both germ and somatic lines. Furthermore, both foxl3 and DMRT1 are expressed in the female KO. The quality of the images figures 6 V to A’ does not allow to define whether there is colocalization within the same cells. Authors should also provide information of KO XY. More specific marker for male or female germinal cells should be used.
Reply: Figure 6 of this study was intended to show the cell fates of female germ cells existed in the early gonads of cyp11a1 mutant XX fish during the transition of ovary development to testis development. These germ cells have two possible fates, either undergo apoptosis or transdifferentiate into male germ cells. Accordingly, we carried out two experiments: 1. Apoptosis analysis. 2. Colocalization analysis of female and male germ cell marker genes by combined whole-mount fluorescence in situ hybridization and immunofluorescence. For apoptosis analyses, given the results of the transcriptome analysis may not be persuasive enough due to the absence of biological replicates, we also performed TUNEL assay. Taken these two results together, we believe that female germ cells in the early gonads of cyp11a1 mutant XX fish did not undergo apoptosis during the transition of ovary development to testis development. In our previous study, we have proved that in tilapia foxl3 is expressed in female germ cells but not in male germ cells. dmrt1 is expressed both in male germ cells and somatic cells but not in female germ cells and somatic cells. The sexual fate of tilapia germ cells is determined by the antagonistic interaction of dmrt1 and foxl3 (Dai et al., 2021). Therefore, we checked the colocalization of foxl3 and dmrt1 in the

early gonads of mutant XX fish. We found some germ cells in the early gonads of cyp11a1 mutant XX fish expressed both foxl3 and dmrt1, and we think that these cells are undergoing transdifferentiation. To date, we haven't identified any other female germ cell or male germ cell marker genes other than foxl3 and dmrt1 in tilapia to distinguish the sexual fate of germ cells in the early gonads. We have improved the images quality of Figure 6V to A', modified the Figure legends (Line 1018-1021) and Discussion section (Line 596-609) in our revised manuscript to make our results clear.
In Figure 6, we would like to show the cell fate of the female germ cells in the early gonads of cyp11a1 mutant XX fish during sex reversal. According to the results shown in Figure 2 and Figure 5, the testicular fate of the cyp11a1 mutant XY fish was not affected, and their gonads directly developed into testes. Therefore, we think it is not necessary to analyze the colocalization of foxl3 and Dmrt1 in the early gonads of the mutant XY fish.
9. The steroid analysis in figure 1 is very interesting and this suggest that there still is some steroids to be produced, while there should not be anything left. Any interpretation, hypothesis to explain this presence while the cyp11a1 KO should be completely devoid of steroid?
Reply: cyp11a1 is the only gene currently known to catalyze the production of P5, the precursor of all steroid hormones. In tilapia, only one cyp11a1 gene exists (Parajes et al., 2013). Therefore, we think that the reason for the presence of some steroids in Figure 1 is more likely due to the non-specificity of the EIA kit. We discussed this in the Discussion section of our revised manuscript (Line 481-486).

Finally, we would like to thank the reviewers for your painstaking efforts in improving the quality of our manuscript. If there are other aspects in the manuscript that require further clarification, kindly let us know and we would be delighted to comply with.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I believe the current paper provides valuable insights on the molecular mechanisms underlying sex differentiation in fish, and will be a useful reference for further research in the area. Sufficient revisions have been made and points have been clarified following the first round of review. I recommend the publication of this work in this journal.

Reviewer #2

(Remarks to the Author)
The present manuscript is a revised version presenting information on the impact of steroid synthesis disruption via CYP11a knock out in tilapia. The authors have appropriately taken into account all comments made by both reviewers and changed the manuscript accordingly. Particularly, the material and methods section was revised to provide necessary details, the results expanded and the discussion particularly modified. I just have 3 very small comments:
- if possible, the authors should add the size of the whole gonads in both male and female (weight and or volume), such that reader will have a broader view of gonadal development (and not "just" the structure)
- I find it somewhat surprising that the CYP11a expression the adult ovary is close to nothing, how do you explain this phenomenon, as adults need steroid production (progesterone-estrogens)?
- Figure 1 Serum P5 and Cort are only presented only in males, why? That would be interesting to show in females as well?

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