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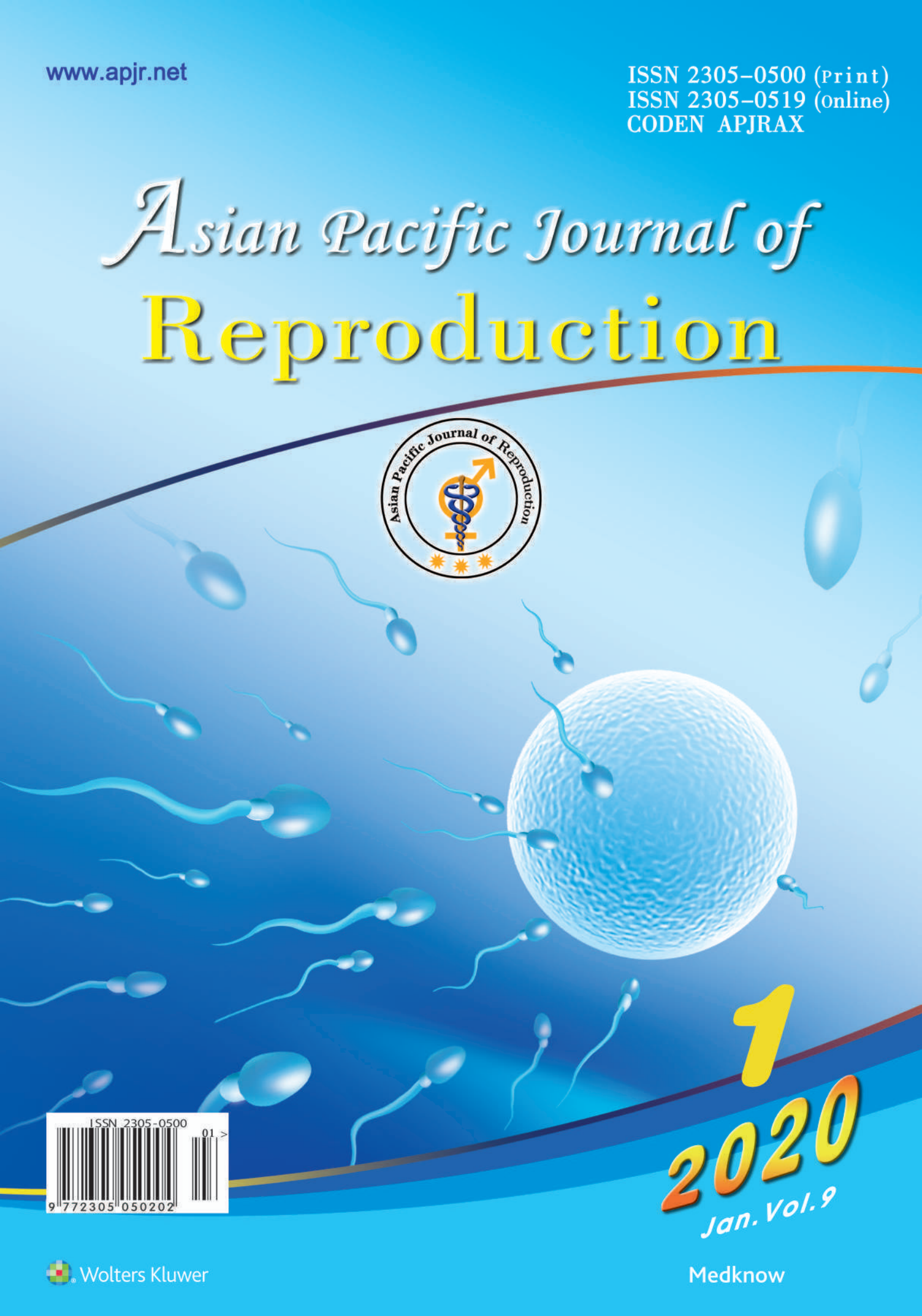

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Dear Miss. Suseno,

NOTE: This e-mail is sent to you as one of the contributing authors. If you are not corresponding author, please co-ordinate with the author designated by your group as the corresponding author for this manuscript

A manuscript has been submitted to our journal Asian Pacific Journal of Reproduction by Dewi Suseno titled '"The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia Oreochromis niloticus".'. A copy of the acknowledgment mail is attached here with for your reference.

Thanking you
Editorial Team
Asian Pacific Journal of Reproduction

Dear Miss. Suseno,

Asian Pacific Journal of Reproduction has received your manuscript entitled ""The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia Oreochromis niloticus"." for consideration for publication. The reference number for this manuscript is "apjr_52_18". Kindly quote this in correspondence related to this manuscript.

The manuscript is being reviewed for possible publication with the understanding that it is being submitted to one journal at a time and have not been published, simultaneously submitted, or already accepted for publication elsewhere either as a whole or in part. Online submission of this article implies that the corresponding author has the written consent from all the contributors to act as corresponding author.

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Covering Letter

To,

The EditorAsian Pacific Journal of Reproduction

Sub: Submission of Manuscript for publication

Dear Sir,

We intend to publish an article entitled **"The impact of sex reversal by oral and immersion methods using 17*α*-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*"** in your journal as an Original Article.

On behalf of all the contributors We will act and guarantor and will correspond with the journal from this point onward.

This manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute's, Universitas Airlangga representative is fully aware of this submission.

This manuscript is original article that we are described from partially thesis study that supported by Ministry of Research, Technology and Higher Education, Republic of Indonesia through Post Doctoral Research Programme. Novelty of this research is examining the residue of methyltestosterone and organ histopathology caused sex reversal application on tilapia fish culture. This manuscript has been corrected and approved by all authors to be published.

We have no conflicts of interest to disclose.

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We would like to suggest following referees for the article, i.e. Penpu Srisakultiew, Department of Fisheries, Khon Kaen University, Thailand (penpusri@gmail.com) and Ihsan Celik, Department of Aquaculture, Faculty of Fisheries, Canakkale Onsekiz Mart University (ihsancelik@comu.edu.tr).

We are highly respected in this Journal, so we submitted this manuscript to this Journal. We hope this manuscript can be immediately evaluated and if possible be accepted for publication in this Journal.

Thank you very much for your consideration of this manuscript.

Best regards,

Dewi Nurmalita Suseno

Corresponding contributor: AkhmadTaufiqMukti

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Contributors' form

Manuscript Title

"The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*".

We certify that we have participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data, as well as the writing of the manuscript, to take public responsibility for it and have agreed to have our name listed as a contributor. we believe the manuscript represents valid work. Neither this manuscript nor one with substantially similar content under our authorship has been published or is being considered for publication elsewhere, except as described in the covering letter. we certify that all the data collected during the study is presented in this manuscript and no data from the study has been or will be published separately. we attest that, if requested by the editors, we will provide the data or will cooperate fully in obtaining and providing the information on which the manuscript is based, for examination by the editors or their assignees. Financial interests, direct or indirect, that exist or may be perceived to exist for individual contributors in connection with the content of this paper have been disclosed in the cover letter. Sources of outside support of the project are named in the cover letter.

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All persons who have made substantial contributions to the work reported in the manuscript, but who are not contributors, are named in the Acknowledgment and have given us their written permission to be named. If we do not include an Acknowledgment that means we have not received substantial contributions from non-contributors and no contributor has been omitted.

Name	Signature	Date signed
1 Dewi Nurmalita Suseno, M.Sc		May, 2. 2018
2 Dr. Epy Muhammad Luqman		May 3. 2018
3 Prof. Mirni Lamid		MAY 11, 2018
4 Dr. Akhmad Taufiq Mukti		May. 16, 2018
5 Prof. Muhammad Agus Supriyadi		May, 10, 2018

Checklist ***(****to be tick marked, as applicable and one copy attached with the manuscript)*

Manuscript Title

"The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*".

Covering letter

- ☑ Signed by all contributors
- ☑ Previous publication / presentations mentioned
- ☑ Source of funding mentioned
- ☑ Conflicts of interest disclosed

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Running title: The impact of sex reversal

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Contribution Details (to be ticked marked as applicable):

	Contributor 1	Contributor 2	Contributor 3	Contributor 4	Contributor 5
Concepts				√	
Design				√	
Definition of intellectual content		√	√	√	√
Literature search	√				
Clinical studies	√				
Experimental studies	√				
Data acquisition				√	
Data analysis	√	√		√	
Statistical analysis			√		
Manuscript preparation	√				
Manuscript editing				√	
Manuscript review		√		√	√
Guarantor					√

Title of the article

"The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*".

Abstract

Objective: To examine sex reversal both by oral and by immersion using 17α-MT on the MT residue concentration and the organ histopathology of tilapia fish. **Methods:** This study was used 3 treatments of 17α-MT administration methods and each treatment was repeated 4 times. Dosages of 17α-MT were used 60 mg/kg feed and 0.5 mL/L media for oral and immersion methods, respectively. First step, larvae were reared at aquaria of 100 L volume, density of 1 fish/L for 2 months. Next steps, fry of tilapia were reared at happa of 2×1×1 m^3 size with density of 30 fish/happa in controlled pond for 3 months. The MT residue concentrations were analyzed by statistical using one-way ANOVA and Duncan's multiple range tests to compared control and treatment groups with the confidence interval $p<0.05$, while organ histopathology was analyzed by descriptive method. **Results:** residue concentrations in serum and residue concentrations in flesh did not exceed the limits of synthetic steroid on the fish body of 5 ng/g. In histopathology organ there were hyperplasia, hypertrophy; clubbing; bending cell haemorrhage; congestion; degeneration of nucleus. infiltrating lymphocytes; neutrophil infiltration; necrosis; inflammation. intestinal villi atrophy; lymphoid follicles; inflammation. **Conclusions:** were obtained that residue concentration of MT still safe. Sex reversal either by oral or by immersion were caused changes on histology of gill, liver, kidneys, and intestine organs.

Keywords: 17α-MT, residue, organ histopathology, tilapia, sex reversal method.

Key Messages:

The use of 17α-methyltestosterone with optimal dose 60 mg/kg feed for oral and 0.5 mL/L for immersion are still safe, relatively

Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be the simple, easy, and highly effective technology [1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT) [2];[3] was a derivative of testosterone [4], which potentially increased sexual developmental in males [3]. The sex reversal of 17α-MT-immersed tilapia larvae produces males of 91.6 - 98.3% [5];[6], however by oral of 60 mg/kg feed produced 93.7% [7], 97.7% [8], even reaches up to 100% of males.

Synthetic steroid hormone would enter through the blood vessels in the body, then it was modulated both by brain and pituitary hormones [9]. Steroid hormone was synthesized either the liver or the kidneys [10], next, it would produce androstenedione which consists both 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sexual, but 17α-MT has characteristic that difficult to absorbed within the body and contaminated the environment [11].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (carcinogenic and endocrine disorder) as well as the danger to the environment [12];[13];[3];[14];[1]. The group of anabolic steroids (including 17α-MT) based on the decision of Minister of Marine Affairs and Fisheries, Republic of Indonesia number KEP.52/MEN/2014 has been banned due to the hormones were harmful for fish, environment and human. Therefore, the aims of research were examined sex reversal both by oral and by immersion using 17α-MT on the MT residue concentration and the organ histopathology of tilapia fish.

Subjects and Methods

Test Animal

Total of tilapia fry that used were 360 fish, which divided into 3 groups and each group was repeated 4 times. Tilapia fry were obtained by artificial fertilization and incubation.

Sex Reversal Treatments

Oral method was conducted since fish of 3 days after hatching (dah) for 28 days, while immersion method was conducted to fish of 10 and 13 dahs for 3 hours, respectively, according to research by Mukti [8] the use 17α-methyltestosterone (Argent) with dose of 60 mg/kg feed and 0.5 mg/L media to oral and immersion methods, respectively.

Rearing of Fish

Initial step, fish was reared at aquaria of 100 L volume, density of 1 fish/L for 2 months. Fish was fed pellet of 40% protein content, 3 times daily at satiation. Sex determination was done through manual observation of fish genetalia and gonad histology preparation in fish sample as much 10 fish for each group. Then, male fish was observed and was selected for maintained further.

Next step, male tilapias of each group were grew separately at happa of 2×1×1 m^3 size with density of 30 fish/hapa in controlled pond for 3 months. Fish was fed pellet of 32% protein content, 3 times daily at-satiation.

Sampling

Fish sampling was done in the 3^{rd}, 4^{th}, and 5^{th} months of each group include control (no treatment of sex reversal) as much 3 fish, respectively to residue test, especially histology preparation, 3-month-old fish was used. Fish was anesthetized using MS222 of 1 mg/L according to Gogal [15], serum (1 mL) was collected according to Atli [16] and flesh (10 g) of fish were collected to do testing of residues using sandwich ELISA method. On the other hand, fish was carefully dissected abdominal part according to research by Wu[17] and organs

of gill, liver, kidneys and intestine were collected and stored in the 50 mL tubes consist buffer neutral formalin (BNF), ratio of 1:2 parts at room temperature before histology preparation.

Measurement of MT Residues

MT residue concentration of sex-reversed tilapia both the serum and the flesh were measured by ELISA method using kit fish methyltestosterone cat number E0103Fi (Bioassay Technology Laboratory, Shanghai, China). Previous, the sample and the reagents were stored at temperature of 18-25^{o}C [3].

Histology Preparation of Organs

Specimens of gill, liver, kidneys, and intestine organs were cut thickness of 2-3 mm, respectively and placed separately in the dish. Histology processes were conducted according standard operational procedure (SOP), generally with slight modified [18].

Results

MT Residue Concentrations

MT concentration of tilapia serum on the 3rd, 4th, and 5th months resulted sex reversal treatment by oral and immersion methods have averages of 5.243 ng/mL and 3.874 ng/mL, respectively and there had a significant difference than control treatment. MT residue concentrations in serum of the male sex-reversed fish was decreased as the fish age, otherwise the normal male was increased every months (Table 1).

On the other hand, MT concentrations of tilapia flesh on 3rd month was no difference between treatments. However, MT concentration of sex-reversed tilapia by either oral or immersion methods on 4th month had 5.995 ng/mL, respectively and there had significant difference compared with normal male (6.259 ng/mL). Based on research was showed that all males, both sex-reversed and normal fish have increased MT residue concentrations in flesh on 5th month. (Table 2).

Organ Histopathology

Treatments of oral and immersion were showed that the gill histolopathology (Fig. 1A) was suffered hyperplasias which found at bottom of the secondary lamella. Hypertrophy appeared on the stem the lamella. This is mainly due to occurrence of containment. Clubbing occurred on the end of primary lamella which caused by the existence of retention, so it happens edema on the lamella. The liver histolopathology (Fig. 1B) showed congestion, haemorrhage and cell atrophy. Congestion was redder because it contained erythrocytes. Haemorrhage was the blood that exit from the centralis. Atrophy showed by the reduction cell size of kupper, which made sinusoid widens and vacuoles degeneration. Congestion caused a sinusoidal filled many erythrocytes that seemed wide. Degeneration of liver cells made enlarged vacuoles. Normally, liver organ did not have damage. Histolopathology of kidney (Fig. 1C) seem haemorrhage, infiltration of lymphocytes and neutrophils, inflammation, and

necrosis. The infiltration presence of lymphocytes and neutrophils had cause inflammation Intestinal histolopathology (Fig. 1D) has look atrophy, intestinal villi haemorrhage, lymphoid follicles, and melanomakrofag. The occurrence of haemorrhage led to the atrophy and melanomakrofag, so there caused erosion and finally, there caused haemorrhage and necrosis of the intestinal villi.

Discussion

Hormonal activities affected by 3 sterochemical aspects, i.e the location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration αorβ, trans and or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It caused by the presence of bacteria in the gastrointestinal tract that oxidize cluster17β-hydroxy to be inactive 17β-keto. Therefore, it necessary to added alkyl group on 17^{th} carbon to become C17α. This was prevent the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17α-MT compound has more activity in the body, but it could caused residue. 17α-MT activity has half the strength of testosterone activity due to the longer of C-chain alkyl groups, and then it would decrease androgenic activity, otherwise it would increased its toxicity.

The 17α-MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts [19];[20].

Exposure of synthetic chemicals and their residue risk for human and wildlife healths [21];[3]. Based on the measure serum MT concentration of 3-months-old male sex-reversed tilapia by oral method had more raising concentration among other treatments. However, on the 4^{th} and 5^{th} months, it was decreased [21]; [3], which states that the MT concentration decreases every months. The fish larvae given MT for 28 days would contain MT only in the first five months after end administration of MT [22].

Testosterone of normal male fish have increased. This was consistent with the study [12], that there were a significant increase in hormone levels in September-October depending on the water temperature and the duration of the dark-light period. This matter was caused the beginning of the spawning season of adult fish. It had increased gene expression from steroidogenic enzymes (P450c17, P450scc, and P450arom), to connected the estradiol and

testosterone during spawning. It was indicated by the increasing of pheromones from androstenedione 50 ng/h until 1 μg/h. The presence of androstenedione may be caused an attraction between fish and it opponent sex which improving the setting of reproductive activity.

The result of measure MT concentration in flesh, fish has higher concentration compared with serum. High enough MT concentration levels were muscle and flesh [23]; [24], concluded that the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT concentration to accumulated in the flesh every months. On the research [24], estimates exogenous steroid remnants of 5 ng/g fish were too low risk to humans. Endogenous testosterone hormone produced on the testes 5.2 ng/g [25], whereas tilapia fish have endogenous testosterone and estradiol hormones of 3 ng/g, respectively [24].

Gill layouts that were outside and directly related to water cause the organs would be the first which affected by the polluted water environment. The food already digested in the intestines would be circulated by blood carried to the liver and kidneys. Liver was the largest organ which responsible of metabolism. Kidneys had functioned as a hyperosmotic regulator [26]. Fish organ taken when the fish was 3 months old, so at that time, the toxicity of 3-month-old fish still appeared.

The early stage of damage caused by gill irritation was accompanied the increasing of the mucous cells at the bottom of epithelial causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached together. Gill lamella looked larger than normal which caused by cell enlargement (hypertrophy) and it looked unclear between the primary and secondary lamellas. According to [16];[27];[28], hyperplasia may occurred due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination characterized by a very dense accumulation of red blood cells (RBCs) in the blood vessels, which would block

blood vessels (congestion), while oedema of lamella looked like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near of the lamella bottom (basal hyperplasia), then the whole room of interlamella filled by new cells which showed like a baseball bat [29];[26].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be return to normal. Necrosis could not be cured, so if it exposed continuously the tissue activity, then would decrease cell activity causing the cells would lose some parts, even causing death. [30];[31]. Congestion preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoidal widen that accumulated blood and haemorrhage. According to the research [29], congestion occurred by the entry of toxic substances into the heart. Haemorrhage was the flow of RBCs out from the central vein.

Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine [32];[33], which cause the area mostly composed by toxic concentration substance causing central venous damage. A sinusoid is a small capillary that separated the fundamental of structural unit with tubule or trabecule (biliary hepatocytes surrounded by a central parenchyma) [32];[34]. Asang fish *Ostechilus hasseltiic* V. which exposed by chemical materials had centralist venous up to 42.70% [33]. Liver had enzyme for drug metabolism which is one of the most damaged organs, but it very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged, it was still capable of regenerating and could even be cured if damage was lost or destroyed [34].

The infected kidneys were swelling which an indication of an inflammatory process that may causing necrosis [35]. Inflammation was an indication of increase lymphocytes and macrophage or neutrophil cells numbers. Kidneys were pollutant-responsive organs to

indicated histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach [36]. Changes that often occured in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression [37];[35];[36].

The intestine damage signed by inflammation. The inflammation or swelling of cells has reversible characteristic, so when it exposed by the toxic substances in a short time period of the cell would return to normal, but if the substance exposed in a long time, then the cell was not able to tolerate damage caused by toxin substances [38]. Melanomacrophage caused by inflammation which followed by erosion of the intestinal villi, haemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would disturb the absorption of important substances, so that fish would suffer from malnutrition. Intestinal organs occurred cell swelling, microvilial cell membrane fused, lysis, intestinal vacuum, intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances [20]. Acute intestinal conditions caused by viruses, parasites, bacteria, algae and intestinal mucosa. Toxic chemicals could be removed using mucous epithelial cells which coiled together with the thickening chromatin and cytoplasmic eosinophils [29]. MT concentrations of serum and flesh have not exceeded the limit due to the estimated residual synthetic steroid in the fish body of 5 ng/g. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are still remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histolopathological figure of the 4- and 5-months-age fish to determine whether there are recovery in fish organ after the cessation of synthetic hormone.

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Title of the article

"The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*".

Abstract

Objective: To examine sex reversal both by oral and by immersion using 17α-MT on the MT residue concentration and the organ histopathology of tilapia fish. **Methods:** This study was used 3 treatments of 17α-MT administration methods and each treatment was repeated 4 times. Dosages of 17α-MT were used 60 mg/kg feed and 0.5 mL/L media for oral and immersion methods, respectively. First step, larvae were reared at aquaria of 100 L volume, density of 1 fish/L for 2 months. Next steps, fry of tilapia were reared at happa of 2×1×1 m^3 size with density of 30 fish/happa in controlled pond for 3 months. The MT residue concentrations were analyzed by statistical using one-way ANOVA and Duncan's multiple range tests to compared control and treatment groups with the confidence interval $p<0.05$, while organ histopathology was analyzed by descriptive method. **Results:** MT residue concentration both serum and flesh of sex-reversed tilapia have significant difference than control. In serum, MT concentration of the male sex-reversed fish was decreased, opposite the normal male was increased every months. On other hand, in flesh, both sex-reversed and normal fish have increased MT residue concentrations on 5^{th} month. Sex reversal caused histopathology in gill, liver, kidney, and intestine organs. **Conclusions:** Sex reversal either by oral or by immersion have MT residu concentration did not exceed the limits of synthetic steroid on the fish body, although their were caused changes on histology of gill, liver, kidneys, and intestine organs.

Keywords: 17α-MT, residue, organ histopathology, tilapia, sex reversal method.

Key Messages:

The use of 17α-methyltestosterone with optimal dose 60 mg/kg feed for oral and 0.5 mL/L for immersion are still safe, relatively

Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be the simple, easy, and highly effective technology [1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT) [2];[3] was a derivative of testosterone [4], which potentially increased sexual developmental in males [3]. The sex reversal of 17α-MT-immersed tilapia larvae produces males of 91.6 - 98.3% [5];[6], however by oral of 60 mg/kg feed produced 93.7% [7], 97.7% [8], even reaches up to 100% of males.

Synthetic steroid hormone would enter through the blood vessels in the body, then it was modulated both by brain and pituitary hormones [9]. Steroid hormone was synthesized either the liver or the kidneys [10], next, it would produce androstenedione which consists both 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sexual, but 17α-MT has characteristic that difficult to absorbed within the body and contaminated the environment [11].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (carcinogenic and endocrine disorder) as well as the danger to the environment [12];[13];[3];[14];[1]. The group of anabolic steroids (including 17α-MT) based on the decision of Minister of Marine Affairs and Fisheries, Republic of Indonesia number KEP.52/MEN/2014 has been banned due to the hormones were harmful for fish, environment and human. Therefore, the aims of research were examined sex reversal both by oral and by immersion using 17α-MT on the MT residue concentration and the organ histopathology of tilapia fish.

Subjects and Methods

Test Animal

Total of tilapia fry that used were 360 fish, which divided into 3 groups and each group was repeated 4 times. Tilapia fry were obtained by artificial fertilization and incubation.

Sex Reversal Treatments

Oral method was conducted since fish of 3 days after hatching (dah) for 28 days, while immersion method was conducted to fish of 10 and 13 days after hatching (dah) for 3 hours, respectively, according to Mukti [8] the use 17α-methyltestosterone (Argent) with dose of 60 mg/kg feed and 0.5 mg/L media to oral and immersion methods, respectively.

Rearing of Fish

Initial step, fish was reared at aquaria of 100 L volume, density of 1 fish/L for 2 months. Fish was fed pellet of 40% protein content, 3 times daily at satiation. Sex determination was done through manual observation of fish genetalia and gonad histology preparation in fish sample as much 10 fish for each group. Then, male fish was observed and was selected for maintained further.

Next step, male tilapias of each group were grew separately at happa of 2×1×1 m^3 size with density of 30 fish/hapa in controlled pond for 3 months. Fish was fed pellet of 32% protein content, 3 times daily at-satiation.

Sampling

Fish sampling was done in the 3^{rd}, 4^{th}, and 5^{th} months of each group include control (no treatment of sex reversal) as much 3 fish, respectively to residue test, especially histology preparation, 3-month-old fish was used. Fish was anesthetized using MS222 of 1 mg/L according to Gogal [15], serum (1 mL) was collected according to Atli [16] and flesh (10 g) of fish was collected to do testing of residues using sandwich ELISA method. On the other hand, fish was carefully dissected abdominal part according to Wu [17] and organs of gill,

liver, kidneys and intestine were collected and stored in the 50 mL tubes consist buffer neutral formalin (BNF), ratio of 1:2 parts at room temperature before histology preparation.

Measurement of MT Residues

MT residue concentration of sex-reversed tilapia both the serum and the flesh were measured by ELISA method using kit fish methyltestosterone cat number E0103Fi (Bioassay Technology Laboratory, Shanghai, China). Previous, the sample and the reagents were stored at temperature of 18-25^{o}C [3].

Histology Preparation of Organs

Specimens of gill, liver, kidneys, and intestine organs were cut thickness of 2-3 mm, respectively and placed separately in the dish. Histology processes were conducted according standard operational procedure (SOP), generally with slight modified [18]. The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

Statistical Analysis

Data of MT residue concentrations were analyzed statistically using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA were followed by Duncan's Multiple Range Test, while organ histopathology was descriptively analyzed.

Results

MT Residue Concentrations

MT concentration of tilapia serum resulted sex reversal treatment both by oral and by immersion methods have significant difference than control treatment on 3, 4 and 5-month-old. MT residue concentration in serum of the male sex-reversed fish was decreased as the fish age, otherwise the normal male was increased every months (Table 1).

On the other hand, MT residue concentration of tilapia flesh on 3rd month was no difference between treatments. However, MT concentration of sex-reversed tilapia either by oral or by immersion on 4 and 5-month-old have significant difference compared with normal male. Based on research was showed that all males, both sex-reversed and normal fish have increased MT residue concentrations in flesh on 5th month (Table 2).

Organ Histopathology

Treatments of oral and immersion were showed that the gill histolopathology (Fig. 1A) was suffered hyperplasias which found at bottom of the secondary lamella. Hypertrophy appeared on the stem the lamella. This is mainly due to occurrence of containment. Clubbing occurred on the end of primary lamella which caused by the existence of retention, so it happens edema on the lamella. The liver histolopathology (Fig. 1B) showed congestion, haemorrhage and cell atrophy. Congestion was redder because it contained erythrocytes. Haemorrhage was the blood that exit from the centralis. Atrophy showed by the reduction cell size of kupper, which made sinusoid widens and vacuoles degeneration. Congestion caused a sinusoidal filled many erythrocytes that seemed wide. Degeneration of liver cells made enlarged vacuoles. Normally, liver organ did not have damage. Histolopathology of kidney (Fig. 1C) seem haemorrhage, infiltration of lymphocytes and neutrophils, inflammation, and necrosis. The infiltration presence of lymphocytes and neutrophils had cause inflammation Intestinal histolopathology (Fig. 1D) has look atrophy, intestinal villi haemorrhage, lymphoid

follicles, and melanomakrofag. The occurrence of haemorrhage led to the atrophy and melanomakrofag, so there caused erosion and finally, there caused haemorrhage and necrosis of the intestinal villi.

Discussion

Hormonal activities affected by 3 sterochemical aspects, i.e the location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration αorβ, trans and or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It caused by the presence of bacteria in the gastrointestinal tract that oxidize cluster17β-hydroxy to be inactive 17β-keto. Therefore, it necessary to added alkyl group on 17^{th} carbon to become C17α. This was prevent the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17α-MT compound has more activity in the body, but it could caused residue. 17α-MT activity has half the strength of testosterone activity due to the longer of C-chain alkyl groups, and then it would decrease androgenic activity, otherwise it would increased its toxicity.

The 17α-MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts [19];[20].

Exposure of synthetic chemicals and their residue risk for human and wildlife healths [21];[3]. Based on the measure serum MT concentration of 3-months-old male sex-reversed tilapia by oral method had more raising concentration among other treatments. However, on the 4^{th} and 5^{th} months, it was decreased [21]; [3], which states that the MT concentration decreases every months. The fish larvae given MT for 28 days would contain MT only in the first five months after end administration of MT [22].

Testosterone of normal male fish have increased. This was consistent with the study [12], that there were a significant increase in hormone levels in September-October depending on the water temperature and the duration of the dark-light period. This matter was caused the beginning of the spawning season of adult fish. It had increased gene expression from steroidogenic enzymes (P450c17, P450scc, and P450arom), to connected the estradiol and

testosterone during spawning. It was indicated by the increasing of pheromones from androstenedione 50 ng/h until 1 μg/h. The presence of androstenedione may be caused an attraction between fish and it opponent sex which improving the setting of reproductive activity.

The result of measure MT concentration in flesh, fish has higher concentration compared with serum. High enough MT concentration levels were muscle and flesh [23]; [24], concluded that the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT concentration to accumulated in the flesh every months. On the research [24], estimates exogenous steroid remnants of 5 ng/g fish were too low risk to humans. Endogenous testosterone hormone produced on the testes 5.2 ng/g [25], whereas tilapia fish have endogenous testosterone and estradiol hormones of 3 ng/g, respectively [24].

Gill layouts that were outside and directly related to water cause the organs would be the first which affected by the polluted water environment. The food already digested in the intestines would be circulated by blood carried to the liver and kidneys. Liver was the largest organ which responsible of metabolism. Kidneys had functioned as a hyperosmotic regulator [26]. Fish organ taken when the fish was 3 months old, so at that time, the toxicity of 3-month-old fish still appeared.

The early stage of damage caused by gill irritation was accompanied the increasing of the mucous cells at the bottom of epithelial causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached together. Gill lamella looked larger than normal which caused by cell enlargement (hypertrophy) and it looked unclear between the primary and secondary lamellas. According to [16];[27];[28], hyperplasia may occurred due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination characterized by a very dense accumulation of red blood cells (RBCs) in the blood vessels, which would block

219 blood vessels (congestion), while oedema of lamella looked like an empty white space that
220 causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near
221 of the lamella bottom (basal hyperplasia), then the whole room of interlamella filled by new
222 cells which showed like a baseball bat [29];[26].

223 Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration
224 was reversible, so when exposed to toxic substances and end administration of MT, cells
225 could be return to normal. Necrosis could not be cured, so if it exposed continuously the
226 tissue activity, then would decrease cell activity causing the cells would lose some parts, even
227 causing death. [30];[31]. Congestion preceded by degeneration of liver cells in which an
228 enlarged vacuole was filled with erythrocytes that cause sinusoidal widen that accumulated
229 blood and haemorrhage. According to the research [29], congestion occurred by the entry of
230 toxic substances into the heart. Haemorrhage was the flow of RBCs out from the central vein.

231 Sinusoidal and central venous damage occurred due to numerous blockages of blood
232 vessels in the stomach and central intestine [32];[33], which cause the area mostly composed
233 by toxic concentration substance causing central venous damage. A sinusoid is a small
234 capillary that separated the fundamental of structural unit with tubule or trabecule (biliary
235 hepatocytes surrounded by a central parenchyma) [32];[34]. Asang fish *Ostechilus hasseltiic*
236 V. which exposed by chemical materials had centralist venous up to 42.70% [33]. Liver had
237 enzyme for drug metabolism which is one of the most damaged organs, but it very resistant to
238 viral or bacterial infections and foreign substances that enter through the absorption in the
239 intestine. It was known that nearly 80% of the liver cells were damaged, it was still capable of
240 regenerating and could even be cured if damage was lost or destroyed [34].

241 The infected kidneys were swelling which an indication of an inflammatory process that
242 may causing necrosis [35]. Inflammation was an indication of increase lymphocytes and
243 macrophage or neutrophil cells numbers. Kidneys were pollutant-responsive organs to

indicated histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach [36]. Changes that often occured in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression [37];[35];[36].

The intestine damage signed by inflammation. The inflammation or swelling of cells has reversible characteristic, so when it exposed by the toxic substances in a short time period of the cell would return to normal, but if the substance exposed in a long time, then the cell was not able to tolerate damage caused by toxin substances [38]. Melanomacrophage caused by inflammation which followed by erosion of the intestinal villi, haemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would disturb the absorption of important substances, so that fish would suffer from malnutrition. Intestinal organs occurred cell swelling, microvilial cell membrane fused, lysis, intestinal vacuum, intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances [20]. Acute intestinal conditions caused by viruses, parasites, bacteria, algae and intestinal mucosa. Toxic chemicals could be removed using mucous epithelial cells which coiled together with the thickening chromatin and cytoplasmic eosinophils [29]. MT concentrations of serum and flesh have not exceeded the limit due to the estimated residual synthetic steroid in the fish body of 5 ng/g. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are still remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histolopathological figure of the 4- and 5-months-age fish to determine whether there are recovery in fish organ after the cessation of synthetic hormone.

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To

The Editor Asian Pacific Journal of Reproduction and Reviewer

Thanks for corrections and suggestions that has been given to our manuscript. Authors responses on corrections and suggestions of reviewer have mentioned in the article with blue-colored words or sentences.

1. Reviewer comment [1]: According to detail of the article, The title should be changed to Residual impact of MT..... (in Title of the article)

   Authors response: We have revised sentences in Title of the article according to Reviewer's suggestion; page 1, line 2-3: "Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia *Oreochromis niloticus*"

2. Reviewer comment [2-5]: about month 3 and 4 (in Abstract of the article)

   Authors response: We have revised sentences in Abstract of the article; page 1, line 15-19: "Residual concentrations in the serum of MT-treated fish indicate lowest and significant difference than normal fish, especially in 4- and 5-month-age tilapias with averages of less than 5 ng/mL, while in normal fish is more than 5 ng/mL. In the flesh, MT residual concentrations showed relatively no significant differences between treatments and MT-treated fish remains lower compared to normal fish, except in 5-month-age tilapia".

3. Reviewer comment [6-7]: about block and fish number (120/treat, 30/replicate) (in Materials and Methods of the article)

   Author response: We have revised sentences in Materials and Methods of the article; page 4, line 94-97: "Next step, a total of male tilapia that used in this study were 360 fish for 3 treatments (120 fish/treatment), then were reared separately at happa of $2\times1\times1m^3$ size in the controlled pond, the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times ".

4. Reviewer comment [8]: The MT oral method will get 0.5 – 1 g (2 – 3 cm) fry while the MT immersion will get only 1 cm fry. So, the test animal in line 75 – 76 would not be enough for Step 1 and 2 (in Materials and Methods of the article)

   Author responses:
   Fish larvae was reared at the same age and long time, for 2 months, only differ in the MT treatment methods. The body weight and total length have same relatively between orally-treated and immersion-treated tilapia fry. The results of sex reversal treatment either by oral or by immersion produce the same survival rate (100%), so that it is still sufficient to be used in the next study step.

   The number of test animal (fish) used in this study is very sufficient for the next treatment step. We also mentioned this in the sub section of Fish Rearing of Materials and Method related to the number of fish seeds reared for 2 months at 100 L-volumed aquaria with a density of 1 fish/L (100 fish/aquarium/replicate/treatment group), as we have stated in Materials and Methods of the article; page 4, line 84-86: "Treatment groups, namely MT-treated fish, both by oral and by immersion and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment".
   On the other hand, the fish that are kept in the next step and used as objects in this study are only male tilapia according to their needs, as we have mentioned in Materials and Methods of

the article; page 4, line 94-95: "Next step, a total of male tilapia that used in this study were 360 fish for 3 treatments (120 fish/treatment),...".

5. Reviewer comment [9]: This reference title is on Triploidy and Sex dimorphism? It is not on sex reversal (in Materials and Methods of the article)

   Authors response: This reference has also been researched and proven about sex reversal in Nile tilapia using 17α-MT which produces about 98% of male sex tilapia, so that the oral and immersion methods of 17α-MT treatment uses this reference to ensure the success and sustainability of this study.

6. Reviewer comment [10]: This is a basic method and unclear sex result (in Materials and Methods of the article)

   Authors responses:
   When the 2-months-old tilapia, the genetalia of fish is visible and relatively easy to distinguish between male and female sex, so that at the age of the fish, male and female sex tilapia can be sorted. To prove the validity of observing the sex differences in fish based on observation of genetalia, it was also verified by gonad determination (gonadal histology) using a simple squash method with acetocarmine staining. This method has been commonly used to prove the sex gonadal characteristics of small-sized fish since the seed.

   We have mentioned sentences in Materials and Methods of the article; page 4, line 90-92: "Sex was determined to 2-month-old fish through manual observation of genitalia for all fish and gonad histology to verified sex of genitalia was obtained to 10 fish/replicate/treatment using the squash method with acetocarmine dye".

7. Reviewer comment [11]: No gonadal histology in result (in Materials and Methods of the article)

   Authors response: In this study, we only use gonadal histology to verify the truth of genetalia observation, not the parameters studied. However, we also attached the images of male and female gonads tilapia on supplementary

8. Reviewer comment [12]: 10/treatment or replicate? Sampling fish number is importance to represent sampling group (in Materials and Methods of the article)

   Authors response: We have revised sentences in Materials and Methods of the article; page 4, line 90-92: "Sex was determined to 2-month-old fish through manual observation of genitalia for all fish and gonad histology to verified sex of genitalia was obtained to 10 fish/replicate/treatment using the squash method with acetocarmine dye".

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   The sex reversal program in tilapia purposes to produce all male tilapia, because male tilapia has a higher growth and body size than female tilapia, so it has great potential and benefits to increase the tilapia production. As we have stated that the sex-reversed tilapia using 17α-MT, both by oral and by immersion methods succeeded to produce male sex tilapia of around 98%,

so it was assumed that no female fish resulted from this treatment. Therefore, this study focused on examining the differences between sex-reversed male tilapia with normal male tilapia (without sex reversal treatment).

We have mentioned sentences in Materials and Methods of the article; page 4, line 93-96: "Then, male fish of 3 treatments were selected for study further.
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Authors responses:
Based on study experience in fish, hormone residue test using the ELISA method in fish aged of under 3 months does not show any real differences. Hormone residues in the fish's body would shows different in fish aged of over 3 months. Is this related to the mechanism of hormone metabolism in the organism (fish) body, we don't know yet. In addition, hormone residues would decrease or disappear in 5-month-old tilapia on cultivated. Therefore, we tried to test the residual concentration in the fish aged of 3 to 5 months.

Indeed, in this study, there are still weaknesses, that is, it has not been able to distinguish between the natural or original MT (endogenous hormone) concentration of the body and the concentration of MT (exogenous hormone) treatment. However, based on the results of this study, it was shown that MT residual concentrations in MT-treated fish, both in the serum and in the flesh were lower and significantly different from normal fish, so we believe that administration of external hormones with certain doses does not produce hormone residues which exceeds hormones of normal fish. **In the future, the suggestions from reviewers to measure the hormone residual concentration at younger fish ages will be considered in future studies.**

Hormone residue test could be done using the ELISA and RIA methods. In more detail, the RIA method using radioactive material is indeed better compared to ELISA method. However, we have limitations for RIA test, specifically the availability of funds. In addition, so far in Indonesia the authority to use RIA equipment is a Nuclear Agency (BATAN), one of the government research institutions, due to the use of radioactive materials, so for this study, we were only able to test using ELISA method. **In the future, reviewers' recommendations regarding the use of radioactive materials for hormone residues test are very much taken into account and consider further studies**.

11. Reviewer comment [15]: 3/treatment? The sampling number is less (in Materials and Methods of the article)

    Authors response: We have revised sentences in Materials and Methods of the article; page 5, line 100: "Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively...".

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    Authors response: In this study, histopathological changes were not performed on gonad organs. Gonad histology of male tilapia (testis) was only performed to observe the spermatozoa production, not histopathological change.

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Thus authors responses on comments, corrections and suggestions of reviewer, we expect a reviewer and editor were pleased and understand it and we hope that this article will be corrected further. Thank you very much.

Best regards,

Akhmad Taufiq Mukti
Corresponding Author: atm_mlg@yahoo.com

Reply to the reviewers' comments

Reviewer Number	Original comments of the reviewer	Reply by the author(s)	Changes done on page number and line number
1	According to detail of the article, The title should be changed to Residual impact of MT..... (in Title of the article)	We have revised sentences in Title of the article according to Reviewer's suggestion; page 1, line 2-3: "Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia *Oreochromis niloticus*"	page 1, line 2-3
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Title of the article

"Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia *Oreochromis niloticus*."

Abstract

Objective: To examine sex reversal both by oral and by immersion using 17α-methyltestosterone on the MT residual concentration and the organ histopathology of tilapia fish. **Methods:** This study was used 2 treatments of sex reversal method and 1 control and each treatment was repeated 4 times. Dosages of 17α-MT were used 60 mg/kg feed and 0.5 mL/L media for oral and immersion methods, respectively. The first step, larvae were reared at 100 L aquaria, the density of 1 fish/L for 2 months. Next steps, male tilapias were reared at happa of 2×1×1 m^3 size in the controlled pond, a density of 30 fish/happa for 3 months. The MT residual concentrations were analyzed by statistical using one-way ANOVA and Duncan's multiple range tests to compared between control and treatments with the confidence interval $p<0.05$, while organ histopathology was analyzed by descriptive method. **Results:** Residual concentrations in the serum of MT-treated fish indicate lowest and significant difference than normal fish, especially in 4- and 5-month-age tilapias with averages of less than 5 ng/mL, while in normal fish is more than 5 ng/mL. In the flesh, MT residual concentrations showed relatively no significant differences between treatments and MT-treated fish remains lower compared to normal fish, except in 5-month-age tilapia. MT-treated tilapia have indicated histopathological changes on gill, liver, kidneys, and intestine organs. **Conclusions:** Sex reversal either by oral or by immersion have MT residual concentration did not exceed the limits of synthetic steroid on the fish body, although their were caused histopathological changes on gill, liver, kidneys, and intestine organs.

Keywords: 17α-MT, residue, organ histopathology, tilapia, sex reversal method.

Key Messages:

The use of 17α-methyltestosterone with an optimal dose of 60 mg/kg feed for oral and 0.5 mL/L for immersion are still safe, relatively.

Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be the simple, easy, and highly effective technology [1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT) [2];[3] is a derivative of testosterone [4], which potentially increased sexual developmental in males [3]. The 17α-MT-immersed tilapia larvae produce males of 91.6 - 98.3% [5];[6], however oral treatment of 60 mg/kg feed produces male of 93.7% [7], 97.7% [8], even reaches up to 100% males.

Synthetic steroid hormone would enter through the blood vessels in the body; then it was modulated by the brain and pituitary hormones [9]. Steroid hormone was synthesized either the liver or the kidneys [10], next, it would produce androstenedione which consists of 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sex, but 17α-MT has characteristic that difficult to absorbed within the body and contaminated the environment [11].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (a carcinogenic and endocrine disorder) as well as the danger to the environment [12];[13];[3];[14];[1]. The group of anabolic steroids (including 17α-MT) based on the decision of Minister of Marine Affairs and Fisheries, Republic of Indonesia number KEP.52/MEN/2014 has been banned due to the hormones were harmful to fish, environment, and human. Therefore, the aims of the study were examined sex reversal both by oral and by immersion using 17α-MT on the MT residual concentration and the organ histopathology changes of tilapia.

Materials and Methods

Test Animal

Test animal that used was Nile tilapia. Tilapia fry were produced by artificial fertilization and controlled incubation..

MT Treatments

MT treatment by oral method was started to 3-days after hatching (dah)-old larvae for 28 days use 17α-MT (Argent) dose of 60 mg/kg feed, while immersion method using dose of 0.5 mg/L was conducted to 10-dah-old fry and repeated in 13-dah-old for 3 hours, respectively according to Mukti [8]. Treatment groups, namely MT-treated fish, both by oral and by immersion and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment.

Fish Rearing

The initial step, fish were reared at 100 L aquaria, the density of 1 fish/L for 2 months, separately each treatment group. Fish was fed commercial pellet content of 40% crude protein, 3 times daily, at-satiation. Sex was determined to 2-month-old fish through manual observation of genitalia for all fish and gonad histology to verified sex of genitalia was obtained to 10 fish/replicate/treatment using the squash method with acetocarmine dye according to Mukti [8]. Then, male fish of 3 treatments were selected for study further.

Next step, a total of male tilapia that used in this study were 360 fish for 3 treatments (120 fish/treatment), then were reared separately at happa of $2\times1\times1m^3$ size in the controlled pond, the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times. Fish was fed commercial pellet content of 32% crude protein, 3 times daily, at-satiation.

Sampling

Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively to residue test, especially histology preparation, 3-month-old fish was used. Fish was anesthetized using MS222 of 1 mg/L according to Gogal [15], serum (1 mL) was collected according to Atli [16], and flesh (10 g) of fish was collected to do testing of residues.

Measurement of MT Residue

MT residue, both the serum and the flesh were measured by the sandwich ELISA method using fish MT kit cat number E0103Fi (Bioassay Technology Laboratory, Shanghai, China). Previously, the sample and the reagents were stored at a temperature of 18-25^{o}C [3].

Histology Preparation

Fish was carefully dissected abdominal part according to Wu [17] and gill, liver, kidneys, and intestine organs were collected and stored in the 50 mL tubes consist buffer neutral formalin (BNF), the ratio of 1:2 parts at room temperature. Histology processes were conducted according to the standard operational procedure (SOP), generally with slight modified [18]. The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

Statistical Analysis

Data of MT residual concentrations were analyzed statistically using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA was followed by Duncan's multiple range test, while organ histopathology was analyzed descriptively.

125 Results

126 **MT Residual Concentrations**

127 MT residual concentration in the serum of MT-treated male tilapia, both by oral and by
128 immersion was decreased as the fish age, while the normal male was increased every month
129 (Table 1). This study indicate lowest (average of less than 5 ng/mL) and significant difference
130 compared to normal male tilapia (average of more than 5 ng/mL) on the 4th and 5th months,
131 except in the 3rd month (oral method) have averages of 5.243±0.080 ng/mL and have
132 significant difference compared to other treatments.

133 In the flesh, MT residual concentration showed relatively no significant difference
134 between treatments. MT-treated male tilapia remains lower than normal male tilapia on the 3rd
135 and 4th months, except in the 5th month. However, the result was showed that all males had
136 increased MT residue on 5th month (Table 2).

137 **Organ Histopathology**

138 MT-treated male tilapias were showed histopathology changes of gill, liver, kidneys, and
139 intestine organs (Fig. 1). In the gill, such as hyperplasia have found to the bottom secondary
140 lamella. Hypertrophy appeared on the lamella stem due to the occurrence of containment.
141 Clubbing occurred on the end of the primary lamella, which caused by the existence of
142 retention, so it happens edema on the lamella (Fig. 1A). The liver was showed congestion,
143 hemorrhage, and cell atrophy (Fig. 1B). Congestion was redder due to contained erythrocytes.
144 Hemorrhage was the blood that exit from the centralis. Atrophy was shown by the reduction
145 cell size of Kupper, which made sinusoid widens and vacuoles degeneration. Congestion was
146 caused sinusoidal filled many erythrocytes that seemed wide. Degeneration of liver cells
147 made enlarged vacuoles. Normally, the liver organ did not have damage. Kidneys seem
148 hemorrhage, infiltration of lymphocytes, and neutrophils, inflammation, and necrosis (Fig.
149 1C). The infiltration presence of lymphocytes and neutrophils had cause inflammation. The

intestine has look atrophy, intestinal villi hemorrhage, lymphoid follicles, and melanomacrophage (Fig. 1D). The occurrence of hemorrhage led to the atrophy and melanomacrophage, so there caused erosion and there caused hemorrhage and necrosis of the intestinal villi, finally.

Discussion

Hormonal activities affected by 3 stereochemical aspects, i.e., location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration αorβ, trans and or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It caused by the presence of bacteria in the gastrointestinal tract that oxidizes cluster 17β-hydroxy to be inactive 17β-keto. Therefore, it necessary to added alkyl group on 17^{th} carbon to become C17α. This prevented the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17α-MT compound has more activity in the body, but it could cause residue. The 17α-MT activity has half the strength of testosterone activity due to the length of C-chain alkyl groups, and then it would decrease androgenic activity. Otherwise, it would increase its toxicity.

The 17α-MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts [19];[20].

Exposure of synthetic chemicals and their residue risk for human and wildlife health [21];[3]. Based on serum MT concentration of male on 3^{rd} month, orally sex-reversed tilapia had more raising concentration among other treatments. However, on the 4^{th} and 5^{th} months, it was decreased, which the MT concentration had decreased every month [21];[3]. The orally MT-treated fish would contain MT only in the initial 5^{th} month [22].

The testosterone of normal male fish has increased. This was consistent with the study [12] that there has a significant increase in hormone levels in September-October depending on the water temperature and the duration of the dark-light period. This matter caused the beginning of the spawning season of adult fish. It had increased gene expression from steroidogenic enzymes (P450c17, P450scc, and P450arom) to connected the estradiol and

testosterone during spawning. The increasing of pheromones indicated it from androstenedione 50 ng/h until 1 μg/h. The presence of androstenedione may be caused by an attraction between fish and its opponent sex which improving the setting of reproductive activity.

MT concentration has higher in the flesh compared to in the serum. High enough MT concentration found in the muscle and flesh [23];[24], because the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT accumulates in the flesh every month. On the research [24], estimates exogenous steroid remnants of 5 ng/g fish were too low risk to humans. Endogenous testosterone hormone produced on the testes 5.2 ng/g [25], whereas tilapia have endogenous testosterone and estradiol of 3 ng/g, respectively [24].

Gill layouts that were outside and directly related to water cause the organs would be the first affected by the polluted water environment. The food already digested in the intestines would be circulated by blood carried to the liver and kidneys. The liver was the largest organ responsible for metabolism. Kidneys had functioned as a hyperosmotic regulator [26]. Fish organ took in 3-months-old, so at that time, the toxicity still appeared.

The early stage of damage caused by gill irritation has accompanied the increasing of the mucous cells at the bottom of epithelial causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached. Gill lamella looked larger than normal which caused by cell enlargement (hypertrophy), and it looked unclear between the primary and secondary lamellas. According to [16];[27];[28], hyperplasia may occur due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination has characterized by a very dense accumulation of red blood cells (RBCs) in the blood vessels, which would block blood vessels (congestion), while edema of lamella looked like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near to the

lamella bottom (basal hyperplasia), then the whole room of interlamellar filled by new cells which showed like a baseball bat [29];[26].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be returned to normal. Necrosis could not be cured, so if it exposed the tissue activity continuously, then would decrease cell activity causing the cells would lose some parts, even causing death. [30];[31]. Congestion preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoidal widen that accumulated blood and hemorrhage. According to the research [29], congestion occurred by the entry of toxic substances into the heart. Hemorrhage was the flow of RBCs out from the central vein.

Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine [32];[33], which cause the area mostly composed by toxic concentration substance causing central venous damage. A sinusoid is a small capillary that separated the fundamental of the structural unit with tubule or trabeculae (biliary hepatocytes surrounded by central parenchyma) [32];[34]. The liver had enzyme for drug metabolism which is one of the most damaged organs, but it very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged; it was still capable of regenerating and could even be cured if the damage was lost or destroyed [33].

The infected kidneys were swelling which an indication of an inflammatory process that may cause necrosis [34]. Inflammation was an indication of increase lymphocytes and macrophage or neutrophil cells numbers. Kidneys were pollutant-responsive organs to indicated histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach [35]. Changes that often occurred in the kidney are inflammation,

necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression [36];[34];[35].

The intestine damage signed by inflammation. The inflammation or swelling of cells has a reversible characteristic, so when it exposed by the toxic substances in a short period of the cell would return to normal, but if the substance exposed in a long time, then the cell was not able to tolerate damage caused by toxin substances [37]. Melanomacrophage caused by inflammation which followed by erosion of the intestinal villi, hemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would disturb the absorption of important substances so that that fish would suffer from malnutrition. Intestinal organs occurred cell swelling, microvilial cell membrane fused, lysis, intestinal vacuum, intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances [20]. Acute intestinal conditions caused by viruses, parasites, bacteria, algae, and intestinal mucosa. Toxic chemicals could be removed using mucous epithelial cells which coiled together with the thickening chromatin and cytoplasmic eosinophils [29]. MT concentrations of serum and flesh have not exceeded the limit due to the estimated residual synthetic steroid in the fish body of 5 ng/g. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histopathological figure of the 4- and 5-months-old fish to determine whether there is a recovery in the fish organ after the cessation of synthetic hormone.

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Covering Letter

To,

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Sub: Submission of Revised Manuscript for Publication

Dear Sir,

We are enclosing herewith a revised manuscript entitled **"The impact of sex reversal by oral and immersion methods using 17*α*-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*"** in your journal for evaluation again.

We have revised this manuscript in accordance suggestions or corrections of reviewer. We hope this revised manuscript can be immediately evaluated again and if possible be accepted for publication in this Journal.

Thank you very much for your consideration of this manuscript.

Best regards,

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Status of the manuscript titled "'The impact of sex reversal by oral and immersion methods using 17α-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia Oreochromis niloticus".' submitted by Miss. Dewi Suseno has been changed and a copy of the mail is as;

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Dear Sir,

We are enclosing herewith a revised manuscript entitled **"Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia *Oreochromis niloticus*"** in your journal for evaluation again.

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Best regards,

Dewi Nurmalita Suseno

Corresponding contributor: Akhmad Taufiq Mukti

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Covering Letter

To,

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Sub: Submission of Revised Manuscript for publication

Dear Sir,

We intend to publish an article entitled **"Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia *Oreochromis niloticus*"** in your journal as an Original Article.

On behalf of all the contributors We will act and guarantor and will correspond with the journal from this point on ward.

This manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute, Universitas Airlangga representative is fully aware of this submission.

This manuscript is original article that we are described from partially thesis study that supported by Ministry of Research, Technology and Higher Education, Republic of Indonesia through Post Doctoral Research Programme. Novelty of this research is examining the residue of methyltestosterone and organ histopathology caused sex reversal application on tilapia fish culture. This manuscript has been corrected and approved by all authors to be published.

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We are highly respected in this Journal, so we submitted this manuscript to this Journal. We hope this manuscript can be immediately evaluated and if possible be accepted for publication in this Journal.

Thank you very much for your consideration of this manuscript.

Best regards,

Dewi Nurmalita Suseno

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1 Dewi Nurmalita Suseno, M.Sc		May, 2. 2018
2 Dr. Epy Muhammad Luqman		May 3. 2018
3 Prof. Mirni Lamid		MAY 11, 2018
4 Dr. Akhmad Taufiq Mukti		May. 16, 2018
5 Prof. Muhammad Agus Supriyadi		May, 10, 2018

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Running title: The impact of sex reversal

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Contribution Details (to be ticked marked as applicable):

	Contributor 1	Contributor 2	Contributor 3	Contributor 4	Contributor 5
Concepts				√	
Design				√	
Definition of intellectual content		√	√	√	√
Literature search	√				
Clinical studies	√				
Experimental studies	√				
Data acquisition				√	
Data analysis	√	√		√	
Statistical analysis			√		
Manuscript preparation	√				
Manuscript editing				√	
Manuscript review		√		√	√
Guarantor					√

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Dear Miss. Suseno
Asian Pacific Journal of Reproduction has received your revised manuscript entitled '"Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia Oreochromis niloticus"..' The manuscript will be re-evaluated by concerned referees for the final decision regarding its suitability for publication. We will get back to you within four weeks.
We thank you for submitting your valuable research work to Asian Pacific Journal of Reproduction.
With warm personal regards,
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3.	Please check the descriptions of the Tables and Figures. And make sure that the descriptions accord with the Tables and Figures.	Has been done	• Table 1, (page number 5, line number 102-103). • Table 2, (page number 5, line number 109). • Pigure 1 (legend figure, original article page number 5-6, line number 112, 116, 122, 124)
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			299, 311, 313, 333).
5.	Please carefully revise the article if there is any mistake.	-	

Covering Letter

To,

The EditorAsian Pacific Journal of Reproduction

Sub: Submission of Revised Manuscript for Publication

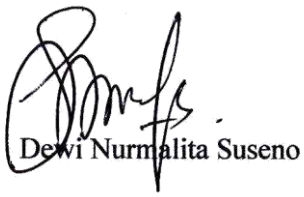
Dear Sir,

We are enclosing herewith a revised manuscript entitled **"The impact of sex reversal by oral and immersion methods using 17*α*-methyltestosterone on methyltestosterone residue and organ histopathology of Nile tilapia *Oreochromis niloticus*"** in your journal for evaluation again.

We have revised this manuscript in accordance suggestions or corrections of reviewer. We hope this revised manuscript can be immediately evaluated again and if possible be accepted for publication in this Journal.

Thank you very much for your consideration of this manuscript.

Best regards,



Corresponding contributor: AkhmadTaufiqMukti

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Dear Miss. Suseno,
The Editorial Board of Asian Pacific Journal of Reproduction is pleased to inform you that your manuscript entitled "Residual impact of methyltestosterone and histopathological changes in sex-reversed Nile tilapia Oreochromis niloticus"., manuscript number apjr_52_18, is accepted for publication in the Journal.
We will be sending you page proofs through the manuscript management site before publication of the manuscript. At that time, you may place the order for extra reprints.
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With warm personal regards,
Yours sincerely,
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Dear authors,

Thanks for submitting your manuscript to the journal. I am pleased to inform you that your paper has been accepted for publication in our journal. Thank you for your contributions. This acceptance is conditional pending final check including quality of figures and ethical approval statement. Please promote your paper by tweeting your paper in social media and also citing your paper in your other publications. We hope you will continue to consider our journal for your future publications.

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Bo Cui, MD, PhD

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Dear Dr. Akhmad Taufiq Mukti,

We have edited your manuscript apjr_52_18. And we need you to revise and improve it well before publication.

Main revision requirements:

1. In the introduction part, since the 17α-MT is banned, why did you want to conduct this study? Does the Minister of Marine Affairs and Fisheries, Republic of Indonesia totally ban the use of 17α-MT? Or can the 17α-MT be used in a normal range of dosage? (Did the Minister rule the maximum dose/limit?)

2. Do the dosage of 60 mg/kg and 0.5 mg/L exceed the limit dose? Do they surpass the maximun dose that Minister of Marine Affairs and Fisheries rules?

3. Are the total fish 900?
Actually, you need to state the number of the fish used in the study in the beginning.
Also, you need yo provide a flow chart about the each step of fish rearing. In each step and each treatment, the number of fish should be specified.

4. Why did you only use 10 fish for verify the sex in each replicate/treatment? Do the 10 fish represent the full sample?

Besides, in the “Results” part, you did not provide the results of sex verification of fish. Actually, you should add the results of sex verification of fish.

5. Provide a flow chart of the treatment of each step and fish rearing.

6. In the “Results” part, the outcome of gonad histology is missing.

7. In the “Discussion part” ,why did you discuss the testosterone since you did not test the testosterone in the study?

8. What is the limit of MT residual concentration?

9. Please add the author contributions in the text.

10. In Table 1, why are the values of normal fish higher than the MT-treated fish?

11. In the “Astract”, please re-organise the results as per the text.

12. Please remove grammatical mistakes in the full text. . Some sentences are really awkward. Please make them easy to undestand.

13. Other revision suggestions and questions are also raised in the text.

Please carefully revise and improve the manuscript. All your revisions should be marked in color. By the way, please do not delete the postils about the revision requirements. Thank you!

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By the way, please do not delete the postils about the revision requirements. Thank you!
Please try to send back your revised manuscript to us before 17th December, 2019. Thank you!

Best regards,
Editor Lin
--

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Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)

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Commented [xb21cn1]:
Main revision requirements:
1. In the introduction part, since the 17α-MT is banned, why did you want to conduct this study? Does the Minister of Marine Affairs and Fisheries, Republic of Indonesia **totally** ban the use of 17α-MT? Or can the 17α-MT be used in a normal range of dosage? (Did the Minister rule the maximum dose/limit?)

2. Do the dosage of 60 mg/kg and 0.5 mg/L exceed the limit dose? Do they surpass the maximun dose that Minister of Marine Affairs and Fisheries rules?

3. Are the total fish 900?
Actually, you need to state the number of the fish used in the study in the beginning.
Also, you need yo provide a **flow chart** about the each step of fish rearing. In each step and each treatment, the number of fish should be specified.

4. Why did you only use 10 fish for verify the sex in each replicate/treatment? Do the 10 fish represent the full sample?

Besides, in the "Results" part, you did not provide the results of sex verification of fish. Actually, you should add the results of sex verification of fish.

5. Provide **a flow chart** of the treatment of each step and fish rearing.

6. In the "Results" part, the outcome of gonad histology is missing.

7. In the "Discussion part" ,why did you discuss the testosterone since you did not test the testosterone in the study?

8. What is the limit of MT residual concentration?

9. Please add the author contributions in the text.

10. In Table 1, why are the values of normal fish higher than the MT-treated fish?

11. In the "Astract", please re-organise the results as per the text.

12. Please remove grammatical mistakes in the full text. . Some sentences are really awkward. Please make them easy to undestand.

13. Other revision suggestions and questions are also raised in the text.

Please carefully revise and improve the manuscript. All your revisions should be marked in color. By the way, please **do not** delete the postils about the revision requirements. Thank you!

Editor Lin
2019.12.11

Abstract

Objective: To examine sex reversal both by oral and by immersion using 17α-methyltestosterone on the methyltestosterone residual concentration and the organ histopathology of tilapia fish.

Methods: This study used oral and immersion treatment methods for sex reversal of tilapia fish and used normal fish as control and each treatment was repeated 4 times. Dosages of 17α-methyltestosterone 60 mg/kg feed and 0.5 mL/L of 17α-methyltestosterone were used for oral and immersion methods, respectively. In the first step, tilapia fry were reared at 100 L aquaria, with a density of 1 fish/L for 2 months. In the next step, male tilapias were reared at happa of (2×1×1) m^3 size in the controlled pond, with a density of 30 fish/happa for 3 months. The methyltestosterone residual concentrations were analyzed by one-way analysis of variance and Duncan's multiple range tests, while organ histopathology was analyzed by descriptive method.

Results: Residual concentrations in the serum of methyltestosterone-treated fish indicated lowest and significant difference than normal fish, especially in 4- and 5-month-age tilapias with averages of less than 5 ng/mL. In the flesh, methyltestosterone residual concentrations showed relatively no significant differences between treatments and methyltestosterone-treated fish remained lower compared to normal fish, except in 5-month-age tilapia. Methyltestosterone-treated tilapia indicated histopathological changes on gill, liver, kidneys, and intestine organs.

Conclusions: Sex reversal either by oral or by immersion has methyltestosterone residual concentration did not exceed the limits of synthetic steroid on the fish body, although methyltestosterone causes histopathological changes on gill, liver, kidneys, and intestine organs.

Commented [xb21cn2]: ??
What is it?

Commented [xb21cn3]: Please re-organise the results as per the text.

Commented [xb21cn4]: What is the limit of MT residual concentration?

Keywords:

17α-methyltestosterone

Residue

Organ histopathology

Tilapia

Sex reversal method

1. Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be a simple, easy, and highly effective technology[1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT)[2,3] is a derivative of testosterone[4], which potentially increases sexual developmental in males[3]. The 17α-MT-immersed tilapia larvae produce males of 91.6%-98.3%[5,6], however, oral treatment of 60 mg/kg feed produces males of 93.7% [7], 97.7%[8], even reaches up to 100% males.

Commented [xb21cn5]: Please cite reference to support the statement.

Synthetic steroid hormone would enter through the blood vessels in the body and then it was modulated by the brain and pituitary hormones[9]. Steroid hormone was synthesized in either the liver or the kidneys[10], and subsequently, it would produce androstenedione which consists of 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sex, but 17α-MT has characteristic that it is difficult to be absorbed within the body and it will also contaminate environment[11].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (a carcinogenic and endocrine disorder) as well as the danger to environment[1,3,12-14]. The group of anabolic steroids (including 17α-MT) based on the decision of Minister of Marine Affairs and Fisheries, Republic of Indonesia (number KEP.52/MEN/2014) has been banned due to the hormones were harmful to fish, environment, and human. Therefore, the aims of the study were to examine sex reversal both by oral and by immersion using 17α-MT on the MT residual concentration and the organ histopathology changes of tilapia.

Commented [xb21cn6]:
Since the 17α-MT is banned, why did you want to conduct this study?

Does the Minister of Marine Affairs and Fisheries, Republic of Indonesia **totally** ban
the use of 17α-MT? Or can the 17α-MT be used in a normal range of dosage? (Did the Minister rule the maximum dose/limit?)

2. Materials and methods

2.1. Test animal

The test animal used was Nile tilapia (*Oreochromis niloticus*). Tilapia fry were produced by artificial fertilization and controlled incubation.

2.2. MT Treatments

MT treatment by oral method was started 3 days after hatching with using 17α-MT (Argent) dose of 60 mg/kg feed. The oral treatment method lasted for 28 days. Immersion method using dose of 0.5 mg/L of 17α-MT was conducted to 10-day-old Tilapia fry and repeated in 13-day-old Tilapia fry for 3 h, respectively[8]. Treatment groups (namely MT-treated fish, both by oral and by immersion) and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment.

Commented [xb21cn7]:
Do the dosage of 60 mg/kg and 0.5 mg/L exceed the limit dose? Do they surpass the maximun dose that Minister of Marine Affairs and Fisheries rules?

How to determine the dose of 17α-MT?

Commented [xb21cn8]: Why not use 3-day-old fry for immersion as the oral treatment method?

Commented [xb21cn9]:
Are the total fish 900?
Actually, you need to state the number of the fish used in the study in the beginning.

Also, you need yo provide a flow chart about the each step of fish rearing. In each step and each treatment, the number of fish should be specified.

2.3. Fish rearing

In the initial step, fish were reared at 100 L aquaria, with a density of 1 fish/L for 2 months, separately in each treatment group. Fish was fed on commercial pellet content of 40% crude protein, 3 times daily, at satiation. Sex was determined on 2-month-old fish through manual observation of genitalia for all fish, and gonad histology. To verify the sex, genitalia was obtained from 10 fish/replicate/treatment by using the squash method with acetocarmine dye according to Mukti[8]. Then, male fish of 3 treatments were selected for further study.

In the next step, a total of 360 male tilapias used in this study for 3 treatments (120 fish/treatment) were reared separately at happa of (2×1×1) m^3 size in the controlled pond, with the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times. Fish was fed on commercial pellet content of 32% crude protein, 3 times daily, at-satiation.

2.4. Sampling

Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively for residue test. 3-month-old fish were used for histology preparation. Fish were anesthetized by using MS222 of 1 mg/L according to Gogal *et al*[15]. Serum (1 mL) was collected according to Atli *et al*[16], and flesh (10 g) of fish was collected to do testing of residues.

2.5. Measurement of MT residue

Commented [xb21cn10]: You need yo provide a flow chart about the each step of fish rearing. In each step and each treatment, the number of fish should be specified.

Commented [xb21cn11]: This is a basic method and unclear sex result.

Commented [xb21cn12]: In the "Results" part, The outcome of gonad histology is missing.

Commented [xb21cn13]:
1. Why did you only use 10 fish for verify the sex in each replicate/treatment? Do the 10 fish represent the full sample?

2. In the "Results" part, you did not provide the results of sex verification of fish. Actually, you should add the results of sex verification of fish.

Commented [xb21cn14]:
Why did you only choose male fish?

If you want to see impact of MT, no need to select only male. Because after MT treatment either oral or immersion, the MT affected the fish fry both male and female. So, you should follow up both sex rather than only male.

Commented [xb21cn15]: happa?

Commented [xb21cn16]: 4 times?
But, in the last paragraph, treatment was repeated 3 times .

MT residue, both the serum and the flesh were measured by the sandwich enzyme-linked immunosorbent assay method using fish MT kit (cat number E0103Fi; Bioassay Technology Laboratory, Shanghai, China). Previously, the sample and the reagents were stored at a temperature of 18-25 ^{o}C[3].

2.6. Histology preparation

Commented [xb21cn17]:
Why is there no gonad histology?

Fish was carefully dissected on abdominal part according to Wu *et al*[17] and gill, liver, kidneys, and intestine organs were collected and stored in the 50 mL tubes which consisted of buffer neutral formalin, with the ratio of 1:2 at room temperature. Histology processes were conducted according to the standard operational procedure, generally with slight modification[18].

2.7. Statistical analysis

Data of MT residual concentrations were analyzed statistically by using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA was followed by Duncan's multiple range test, while organ histopathology was analyzed descriptively. Data were expressed as mean ± standard deviation (mean ±SD). *P*-value < 0.05 was considered as statistical difference.

2.8. Ethical approval

The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

3. Results

3.1. MT residual concentrations

MT residual concentration in the serum of MT-treated male tilapia, both by oral and by immersion, was decreased on 4th month while increased again in 5th month, the normal male fish was increased from 3rd month through out the 5th month (Table 1). On 4th and 5th months, the MT residue concentrations were lower in both oral and immersion groups comparing with that of the normal group （*P* both <0.05）.

In the flesh, MT residual concentration showed relatively no significant difference between the oral and immersion treatment groups. MT-treated male tilapia remained lower than normal male tilapia in the 3rd and 4th months, except in the 5th month. However, the result showed that all males had increased MT residue in the 5th month comparing with that of the 4th months(Table 2).

3.2. Organ histopathology

MT-treated male tilapias showed histopathology changes in gill, liver, kidneys, and intestine organs (Figure 1). In the gill, such as hyperplasia was found in the bottom secondary lamella. Hypertrophy appeared on the lamella stem due to the occurrence of containment.

Commented [xb21cn18]: Since the normal fish did not receive MT, why did the MT residue concentrations in normal fish increase with age increasing?

Commented [xb21cn19]: Why are the values of normal fish higher than the MT-treated fish?

Clubbing occurred at the end of the primary lamella, which was caused by the existence of retention, so edema appeared on the lamella (Figure 1A). The liver showed congestion, hemorrhage, and cell atrophy (Figure 1B). Congestion was redder due to contained erythrocytes. ~~Hemorrhage was the blood that exit from the centralis.~~ Atrophy was shown by the reduction cell size of Kupper, which made sinusoid widen and made vacuoles degenerate. Congestion caused sinusoidal erythrocytes to wide. Degeneration of liver cells made vacuoles enlarge. Normally, the liver organ did not have damage. Kidneys seem hemorrhage, infiltration of lymphocytes, and neutrophils, inflammation, and necrosis (Figure 1C). The infiltration presence of lymphocytes and neutrophils caused inflammation. The intestine has look atrophy, intestinal villi hemorrhage, lymphoid follicles, and melanomacrophage (Figure 1D). The occurrence of hemorrhage led to the atrophy and melanomacrophage, so finally, it caused erosion and hemorrhage and necrosis of the intestinal villi.

4. Discussion

Commented [xb21cn20]: Please add limitation of the study.

Hormonal activities are affected by three stereochemical aspects, *i.e.*, location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration α or β, trans and/or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It is caused by the presence of bacteria in the gastrointestinal tract that oxidizes cluster 17β-hydroxy to be inactive 17β-keto. Therefore, it is necessary to add alkyl group on 17th carbon to become C17α. This prevented the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17α-MT compound has more activity in the body, but it could cause residue. The 17α-MT activity has half the strength of testosterone activity due to the length of

C-chain alkyl groups, and then it would decrease androgenic activity. Otherwise, it would increase its toxicity.

The 17α-MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts[19,20].

Exposure to synthetic chemicals and their residue is risk for human and wildlife health[3,21]. Based on serum MT concentration of males on the 3rd month, orally sex-reversed tilapia had more raising concentration than other treatments. However, in the 4th and 5th months, the MT concentration had decreased every month[3,21]. The orally MT-treated fish would contain MT only in the initial 5 months[22].

The testosterone of normal male fish has increased. This was consistent with the study of Khalil *et al*[12] that there was a significant increase in hormone levels in September and October depending on the water temperature and the duration of the dark-light period. This matter caused the beginning of the spawning season of adult fish. It had increased gene expression from steroidogenic enzymes (P450c17, P450scc, and P450arom) to connect the estradiol and testosterone during spawning. The increasing of pheromones indicated it from androstenedione 50 ng/h until 1 μg/h. The presence of androstenedione may be caused by an attraction between fish and its opponent sex which improves the setting of reproductive activity.

Commented [xb21cn21]: But in your study, you did not test the testosterone.
Why did you discuss the testosterone?

MT concentration was higher in the flesh compared to in the serum. High enough MT concentration was found in the muscle and flesh[23,24], because the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT to accumulate in the flesh every month. In the research of Pandian and Kirankumar[24], exogenous steroid remnants of 5 ng/g in fish were too low risk to humans. Endogenous testosterone hormone produced on

the testes was 5.2 ng/g[25], whereas tilapia had endogenous testosterone and estradiol of 3.0 ng/g, respectively[24].

Commented [Yan 22]: Please use **ng/kg** instead of ng/g. Please re-calculate the values of hormone.

Gill layouts that were outside and directly related to water ~~cause the organs~~ would be the first affected by the polluted water environment. The food already digested in the intestines would be circulated by blood to the liver and kidneys. The liver was the largest organ responsible for metabolism. Kidneys had functioned as a hyperosmotic regulator[26]. Fish organ took in 3-months-old, so at that time, the toxicity still appeared.

Commented [xb21cn23]: ???
Confusing! Actually, you need to describe the full sentence clearly.

The early stage of damage caused by gill irritation has accompanied the increasing of the mucous cells at the bottom of epithelia with causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached. Gill lamella looked larger than normal which was caused by cell enlargement (hypertrophy), and it looked unclear between the primary and secondary lamellas. According to previous studies[16,27,28], hyperplasia may occur due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination has characterized by a very dense accumulation of red blood cells in the blood vessels, which would block blood vessels (congestion), while edema of lamella looks like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near to the lamella bottom (basal hyperplasia), and then the whole room of interlamellar was filled by new cells which showed like a baseball bat[26,29].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be returned to normal. Necrosis could not be cured, so if it exposed the tissue activity continuously, then it would decrease cell activity, causing the cells to lose some parts even to death[30,31]. Congestion was preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoid widen that accumulated blood and

hemorrhage. According to the research of Robert[29], congestion occurred by the entry of toxic substances into the heart. Hemorrhage was the flow of red blood cells out of the central vein.

Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine[32,33], which cause the area mostly to be composed by toxic concentration substances, causing central venous damage. A sinusoid is a small capillary that separated the fundamental of the structural unit with tubule or trabeculae (biliary hepatocytes surrounded by central parenchyma)[32,34]. The liver had enzyme for drug metabolism which is one of the most damaged organs but is very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged. But, it was still capable of regenerating and could even be cured if the damage was lost or destroyed[33].

Commented [xb21cn24]: Confusing! Actually, you need to describe the full sentence clearly.

The infected kidneys were swelling, which was an indication of an inflammatory process that may cause necrosis[34]. Inflammation was an indication of increased lymphocytes and macrophage or neutrophil cell numbers. Kidneys were pollutant-responsive organ to indicate histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach[35]. Changes that often occurred in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression[34-36].

The intestine damage is signed by inflammation. The inflammation or swelling of cells has a reversible characteristic that exposed to the toxic substances in a short period, the cell would return to normal, but if exposed to the toxic substances for a long time, the cell was not able to tolerate damage caused by toxin substances[37]. Melano-macrophage was caused by inflammation which was followed by erosion of the intestinal villi, hemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would

disturb the absorption of important substances so that that fish would suffer from malnutrition. In intestinal organs, there were cell swelling, microvilial cell membrane fused, lysis, intestinal vacuum and intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances[20]. Acute intestinal conditions were caused by viruses, parasites, bacteria, algae, and intestinal mucosa. Toxic chemicals could be removed by using mucous epithelial cells that coiled together with the thickening chromatin and cytoplasmic eosinophils[29]. MT concentrations of serum and flesh have not exceeded the limit due to the estimated residual synthetic steroid in the fish body of 5 ng/g. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histopathological figure of the 4- and 5-month-old fish to determine whether there is a recovery in the fish organ after the cessation of synthetic hormone.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Author contributions

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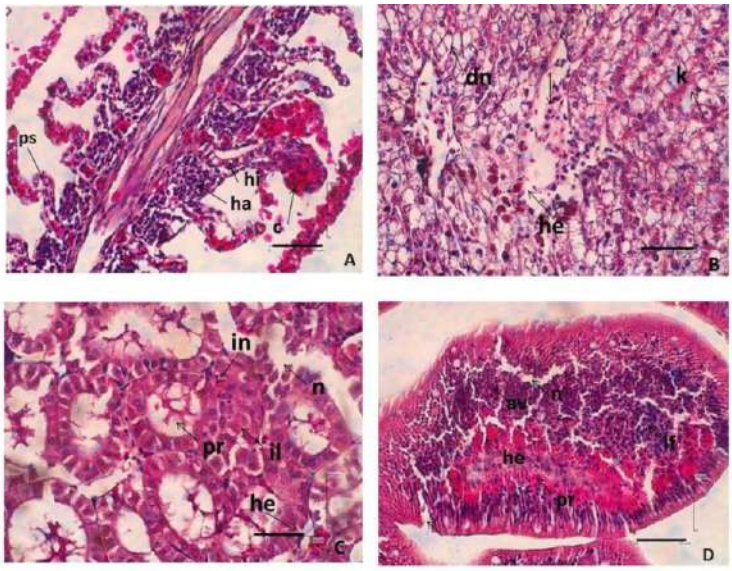

Figure 1. Organ histopathology of 3-month-old male tilapia fish (*n* = 3) (H&E staining, scale bar = 50 μm). (A) gill, (B) liver, (C) kidney, and (D) intestine. Note: ha = hyperplasia; hi = hypertrophy; c = clubbing; ps = bending cell; he = haemorrhage; k = congestion; dn = degeneration of nucleus; il = infiltrating lymphocytes; in = neutrophil infiltration; n = necrosis; pr = inflammation; av = intestinal villi atrophy; lf = lymphoid follicles; pr = inflammation.

Table 1. MT residue concentrations (ng/mL) of serum in different age of male tilapia.

Treatments	Ages of tilapia (month)		
	3	4	5
Normal	4.403 ± 0.058 [a]	5.117 ± 0.057 [c]	5.105 ± 0.079 [c]
Oral	5.243 ± 0.080 [b]	4.171 ± 0.051 [b]	4.266 ± 0.050 [a]
Immersion	4.431± 0.029 [a]	3.874 ± 0.038 [a]	4.450 ± 0.054 [b]

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

Commented [xb21cn36]: Question:

Since the normal fish did not receive MT, why did the MT residue concentrations in normal fish increase with age increasing?

Commented [xb21cn37]: Why are the values of normal fish higher than the MT-treated fish?

Table 2. MT residue concentrations (ng/g) of the flesh in different age of male tilapia.

Treatments	Ages of tilapia (month)		
	3	4	5
Normal	6.061 ± 0.094 [a]	6.259 ± 0.088 [b]	6.272± 0.109 [a]
Oral	5.967 ± 0.058 [a]	5.995 ± 0.079 [a]	7.099± 0.135 [b]
Immersion	5.900 ± 0.100 [a]	5.898 ± 0.079 [a]	6.403 ± 0.088 [a]

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

Commented [xb21cn38]: Since the normal fish did not receive MT, why did the MT residue concentrations in normal fish increase with age increasing?

Reply to the editor's or reviewers' comments

Reviewer Number	Original comments of the reviewer	Reply by the author(s)	Changes done on page number and line number
1	In the introduction part, since the 17α-MT is banned, why did you want to conduct this study? Does the Minister of Marine Affairs and Fisheries, Republic of Indonesia totally ban the use of 17α-MT? Or can the 17α-MT be used in a normal range of dosage? (Did the Minister rule the maximum dose/limit?)	We have mentioned some sentences in the Introduction of the article 1. We conducted this study aimed: a) to prove the presumption that has been the subject of debate in the fish farmer community that the use of 17α-MT at any dose produces dangerous and toxic residues when consumed by humans and the released into the environment, as well as the debate among researchers as we have mentioned in the Introduction of the article b) to address concerns that have existed in the community and policy makers (in this case the government i.e. Ministry of Marine Affairs and Fisheries, Republic of Indonesia) that the use of 17α-MT in certain doses is still safe and does not contain dangerous residues of concern so far. 2. Whereas, 17α-MT in some countries is still used in the fish farming, especially to produce male monosex tilapia because male tilapia has faster	

		growth than female tilapia, with the requirement that the dosage used is optimal and safe, both for the humans and the environment. 3. Therefore, we hope the results of this study can be a recommendation for the government, specifically Ministry of Marine Affairs and Fisheries, Republic of Indonesia that 17α-MT in optimal and safe doses can still be used in fish farming, especially for the production of male monosex in fish with maximum results, so that the regulation can be revised again for the advancement of aquaculture in Indonesia while maintaining a sustainable environment and human health that consumes cultured fish. 4. Decree of the Ministry of Marine Affairs and Fisheries, Republic of Indonesia (Number: KEP.52/MEN/ 2014) stated that to increase aquaculture production that is healthy, quality, safe for consumption, and competitiveness is prohibited from using hormones that are harmful to fish, the environment, and humans who consume this fish. The 17α-MT is one of the hard drugs of the hormone class that is prohibited from being used. Hard drugs are fish medicines which if their use is not in accordance with the provisions can	

		cause danger to fish, environment, and humans. 5. The Decree of the Ministry of Marine Affairs and Fisheries, Republic of Indonesia (Number:KEP.52/MEN/ 2014) does not set a maximum dose and/or limit of 17α-MT.	
2	Do the dosage of 60 mg/kg and 0.5 mg/L exceed the limit dose? Do they surpass the maximun dose that Minister of Marine Affairs and Fisheries rules?	1. The doses of 60 mg/kg and 0.5 mg/L through oral and immersion methods, respectively, are doses that have been widely studied and can produce male monosex in tilapia up to around 98%, as the results of a study conducted by Mukti (2016), as we have mentioned in the article. These doses have been used as a reference and as a method for mass production of male monosex in tilapia. Based on the results of this study also showed that the doses proved to not produce residues that exceed the hormonal residues of normal fish, even showed a relatively lower residual value than normal fish (especially in serum). Even the residue resulting from the use of these doses is still below the residual tolerance threshold that is considered to have too low to be a hazard to human health, which is 5000 ng/L or 5000 ng/kg (Pandian and Kirankumar, 2003). Although, in this study, we have not been able to test and differentiate the content or residue of	

		the MT hormone between the original (endogenous) and the introduction (exogenous or synthetic). 2. The Ministry of Marine Affairs and Fisheries, Republic of Indonesia by Decree Number: KEP.52/MEN/2014 does not set a maximum dose and/or limit in the use of 17α-MT, as well as point 4 in previous author's response above.	
3	Are the total fish 900? Actually, you need to state the number of the fish used in the study in the beginning. Also, you need yo provide a flow chart about the each step of fish rearing. In each step and each treatment, the number of fish should be specified	Yes, we was used a total number of 900 fish larvae or fry, each treatment (oral-treated, immersion-treated, and normal fish) with density of 100 fish and 3 times replication, respectively. Keep in mind that this treatment was conducted to produce male monosex at laboratory scale before main treatment at grow-out period (field scale) that is the focus of this study. We also have mentioned in the Materials and methods of the article. We also have added flow chart in he ends of Materials and methods of article.	
4	Why did you only use 10 fish for verify the sex in each replicate/treatment? Do the 10 fish represent the full sample? Besides, in the "Results" part, you did not provide the results of sex verification of fish. Actually, you should add the results of sex verification of fish.	Ten fish were enough to be used as a histological gonad observation sample because the population we used was homogeneous and the sampling we did was random sampling. We do 10 fish sampling/repetition/treatment. We need to say that the sampling of 10	

		fish for histology of the gonad is only used to verify the sex we have done by visual morphology of genetalia. We want to prove that the morphological verification of genetical sex that we do is completely valid with the support of fish samples for gonad histology. Based on the results of this treatment it is evident that the MT treatment, both orally and soaking yields an average of about 98% males in tilapia. We have mentioned and added the figure of gonad histology using squash method with acetocarmine dye to verify of sex.	
5	Provide a flow chart of the treatment of each step and fish rearing.	We have mentioned and added a flow chart in the ends Materials and methods of the article.	
6	In the “Results” part, the outcome of gonad histology is missing	We have mentioned and added the figures of gonad and testicular histologies	
7	In the “Discussion part” ,why did you discuss the testosterone since you did not test the testosterone in the study?	We have deleted sentences or paragraph in the Discussion of the article.	
8	What is the limit of MT residual concentration?	5000 ng/L or 5000 ng/kg is too low risk for humans health according to Pandian and Kirankumar (2003).	
9	Please add the author contributions in the text.	We have mentioned in the Author contributions of the article.	
10	In Table 1, why are the values of normal fish higher than the MT-treated fish?	Normal fish have higher MT residue value than MT-treated fish. We suspect this is related to the reproductive cycle or	

		period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia. In addition, we have also stated that in this study, we have not been able to test and differentiate the content or residue of MT hormones between the original and those introduced from exogenous (synthetic), so how much is the content or residue of the original MT hormone and the result of introduction, especially in male monosex tilapia from 17α-MT treatment. Therefore, in the future, this limitatation are our concern for further studies.	
11	In the “Abstract”, please re-organise the results as per the text.	We have revised and re-organized sentences in the Abstract, especially the results.	
12	Please remove grammatical mistakes in the full	We have revised and corrected some	

	text. . Some sentences are really awkward. Please make them easy to undestand.	sentences in the article, especially in the Discussion of the article.	
13	Other revision suggestions and questions are also raised in the text	We have revised article based on editor's suggestions and corrections	

Covering Letter

To,

The Editor Asian Pacific Journal of Reproduction

Sub: Submission of Revised Manuscript for publication

Dear Sir,

We intend to publish an article entitled **"Residual impact of 17*α*-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)"** in your journal as an Original Article.

On behalf of all the contributors We will act and guarantor and will correspond with the journal from this point on ward.

This manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute, Universitas Airlangga representative is fully aware of this submission.

This manuscript is original article that we are described from partially thesis study that supported by Ministry of Research, Technology and Higher Education, Republic of Indonesia through Post Doctoral Research Programme. Novelty of this research is examining the residue of methyltestosterone and organ histopathology caused sex reversal application on tilapia fish culture. This manuscript has been corrected and approved by all authors to be published.

We have no conflicts of interest to disclose.

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We would like to suggest following referees for the article, i.e. Penpu Srisakultiew, Department of Fisheries, Khon Kaen University, Thailand (penpusri@gmail.com) and Ihsan Celik, Department of Aquaculture, Faculty of Fisheries, Canakkale Onsekiz Mart University (ihsancelik@comu.edu.tr).

We are highly respected in this Journal, so we submitted this manuscript to this Journal. We hope this manuscript can be immediately evaluated and if possible be accepted for publication in this Journal.

Thank you very much for your consideration of this manuscript.

Best regards,

Dewi Nurmalita Suseno

Corresponding contributor: Akhmad Taufiq Mukti

e-mail: atm_mlg@yahoo.com

Contributors' form

Manuscript Title

"Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)".

We certify that we have participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data, as well as the writing of the manuscript, to take public responsibility for it and have agreed to have our name listed as a contributor. we believe the manuscript represents valid work. Neither this manuscript nor one with substantially similar content under our authorship has been published or is being considered for publication elsewhere, except as described in the covering letter. we certify that all the data collected during the study is presented in this manuscript and no data from the study has been or will be published separately. we attest that, if requested by the editors, we will provide the data or will cooperate fully in obtaining and providing the information on which the manuscript is based, for examination by the editors or their assignees. Financial interests, direct or indirect, that exist or may be perceived to exist for individual contributors in connection with the content of this paper have been disclosed in the cover letter. Sources of outside support of the project are named in the cover letter.

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Name	Signature	Date signed
1 Dewi Nurmalita Suseno, M.Sc		May, 2. 2018
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3 Prof. Mirni Lamid		MAY 11, 2018
4 Dr. Akhmad Taufiq Mukti		May. 16, 2018
5 Prof. Muhammad Agus Supriyadi		May, 10, 2018

Checklist ***(****to be tick marked, as applicable and one copy attached with the manuscript)*

Manuscript Title

"Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)".

Covering letter

- ☑ Signed by all contributors
- ☑ Previous publication / presentations mentioned
- ☑ Source of funding mentioned
- ☑ Conflicts of interest disclosed

Authors

- ☑ Middle name initials provided
- ☑ Author for correspondence, with e-mail address provided
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"Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)".

Running title: The impact of sex reversal

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Contribution Details (to be ticked marked as applicable):

	Contributor 1	Contributor 2	Contributor 3	Contributor 4	Contributor 5
Concepts				√	
Design				√	
Definition of intellectual content		√	√	√	√
Literature search	√				
Clinical studies	√				
Experimental studies	√				
Data acquisition				√	
Data analysis	√	√		√	
Statistical analysis			√		
Manuscript preparation	√				
Manuscript editing				√	
Manuscript review		√		√	√
Guarantor					√

Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)

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Commented [xb21cn1]:
Main revision requirements:
1. In the introduction part, since the 17α-MT is banned, why did you want to conduct this study? Does the Minister of Marine Affairs and Fisheries, Republic of Indonesia **totally** ban the use of 17α-MT? Or can the 17α-MT be used in a normal range of dosage? (Did the Minister rule the maximum dose/limit?)

2. Do the dosage of 60 mg/kg and 0.5 mg/L exceed the limit dose? Do they surpass the maximun dose that Minister of Marine Affairs and Fisheries rules?

3. Are the total fish 900?
Actually, you need to state the number of the fish used in the study in the beginning.
Also, you need yo provide a **flow chart** about the each step of fish rearing. In each step and each treatment, the number of fish should be specified.

4. Why did you only use 10 fish for verify the sex in each replicate/treatment? Do the 10 fish represent the full sample?

Besides, in the "Results" part, you did not provide the results of sex verification of fish. Actually, you should add the results of sex verification of fish.

5. Provide **a flow chart** of the treatment of each step and fish rearing.

6. In the "Results" part, the outcome of gonad histology is missing.

7. In the "Discussion part" ,why did you discuss the testosterone since you did not test the testosterone in the study?

8. What is the limit of MT residual concentration?

9. Please add the author contributions in the text.

10. In Table 1, why are the values of normal fish higher than the MT-treated fish?

11. In the "Astract", please re-organise the results as per the text.

12. Please remove grammatical mistakes in the full text. . Some sentences are really awkward. Please make them easy to undestand.

13. Other revision suggestions and questions are also raised in the text.

Please carefully revise and improve the manuscript. All your revisions should be marked in color. By the way, please **do not** delete the postils about the revision requirements. Thank you!

Editor Lin
2019.12.11

Abstract

Objective: To examine sex reversal both by oral and by immersion using 17α-methyltestosterone on the methyltestosterone residual concentration and the organ histopathology of tilapia fish.

Methods: This study used oral and immersion treatment methods for sex reversal of tilapia fish and used normal fish as control and each treatment was repeated 4 times. Dosages of 17α-methyltestosterone 60 mg/kg feed and 0.5 mL/L of 17α-methyltestosterone were used for oral and immersion methods, respectively. In the first step, tilapia fry were reared at 100 L aquaria, with a density of 1 fish/L for 2 months. In the next step, male tilapias were reared at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with a density of 30 fish/happa for 3 months. The methyltestosterone residual concentrations were analyzed by one-way analysis of variance and Duncan's multiple range tests, while organ histopathology was analyzed by descriptive method.

Results: Residual concentrations in the serum of methyltestosterone-treated fish indicated lowest and significant difference than normal fish, especially in 4- and 5-month-oldage tilapias with averages of less than 5000 ng/mL, while in normal fish is more than 5000 ng/L. In the flesh, methyltestosterone residual concentrations showed relatively no significant differences between treatments and methyltestosterone-treated fish remained lower compared to normal fish, except in 5-month-oldage tilapia. Methyltestosterone-treated tilapia have indicated histopathological changes on gill, liver, kidneys, and intestine organs.

Conclusions: Sex reversal either by oral or by immersion has methyltestosterone residual concentration did not exceed the limits (5000 ng/L or 5000 ng/kg) of synthetic steroid on the fish body, although methyltestosterone causes histopathological changes on gill, liver, kidneys, and intestine organs.

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Commented [xb21cn2]: ??
What is it?

Authors response: happa is net cage
We have mentioned in the text.

Commented [xb21cn3]: Please re-organise the results as per the text.

Authors response: We have revised and re-organized the results as per text.

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Commented [xb21cn4]: What is the limit of MT residual concentration?

Authors response: The limit of MT residual concentration is based on according to Pandian and Kirankumar [25] stated that residual steroid of 5000 ng/kg fish is too
low to be a hazard to human health.

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Keywords:

17α-methyltestosterone

Residue

Organ histopathology

Tilapia

Sex reversal method

1. Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be a simple, easy, and highly effective technology [1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT) [2,3] is a derivative of testosterone [4], which potentially increases sexual developmental in males [3]. The 17α-MT-immersed tilapia larvae produce males of 91.6%-98.3% [5,6], however, oral treatment of 60 mg/kg feed produces males of 93.7% [7], 97.7% [8], even reaches up to 100% males [9].

Synthetic steroid hormone would enter through the blood vessels in the body and then it was modulated by the brain and pituitary hormones [109]. Steroid hormone was synthesized in either the liver or the kidneys [110], and subsequently, it would produce androstenedione which consists of 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sex, but 17α-MT has characteristic that it is difficult to be absorbed within the body and it will also contaminate environment[121].

Commented [xb21cn5]: Please cite reference to support the statement.

Authors response: We have sited of references [9] and we have mentioned in the references of the article.

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The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (a carcinogenic and endocrine disorder) as well as the danger to environment [1,3,132-154]. The group of anabolic steroids (including 17α-MT) based on the decision of Minister of Marine Affairs and Fisheries, Republic of Indonesia (number KEP.52/MEN/2014) has been banned due to the hormones were harmful to fish, environment, and human. This study expected to prove the presumption that has been the subject of debate in the fish farmer community that the use of 17α-MT at any dose produces dangerous and toxic residues when consumed by humans and the released into the environment, as well as the debate among researchers and to address concerns that have existed in the community and policy makers that the use of 17α-MT in certain doses is still safe and does not contain dangerous residues of concern so far. So that the regulation can be revised again for the advancement of aquaculture while maintaining a sustainable environment and human health that consumes cultured fish. Therefore, the aims of the study were to examine sex reversal both by oral and by immersion using 17α-MT on the MT residual concentration and the organ histopathology changes of tilapia.

Commented [xb21cn6]:
Since the 17α-MT is banned, why did you want to conduct this study?

Authors responses:
1.We conducted this study aimed:
a)to prove the presumption that has been the subject of debate in the fish farmer community that the use of 17α-MT at any dose produces dangerous and toxic residues when consumed by humans and the released into the environment, as well as the debate among researchers as we have mentioned in the Introduction of the article
b)to address concerns that have existed in the community and policy makers (in this case the government i.e. Ministry of Marine Affairs and Fisheries, Republic of Indonesia) that the use of 17α-MT in certain doses is still safe and does not contain dangerous residues of concern so far.
2.Whereas, 17α-MT in some countries is still used in the fish farming, especially to produce male monosex tilapia because male tilapia has faster growth than female tilapia, with the requirement that the dosage used is optimal and safe, both for the humans and the environment.
3.Therefore, we hope the results of this study can be a recommendation for the government, specifically Ministry of Marine Affairs and Fisheries, Republic of Indonesia that 17α-MT in optimal and safe doses can still be used in fish farming, especially for the production of male monosex in fish with maximum results, so that the regulation can be revised again for the advancement of aquaculture in Indonesia while maintaining a sustainable environment and human health that consumes cultured fish.

Does the Minister of Marine Affairs and Fisheries, Republic of Indonesia **totally** ban
the use of 17α-MT? Or can the 17α-MT be used in a normal range of dosage? (Did the Minister rule the maximum dose/limit?)

Authors responses:
Decree of the Ministry of Marine Affairs and Fisheries, Republic of Indonesia (Number: KEP.52/MEN/ 2014) stated that to increase aquaculture production that is healthy, quality, safe for consumption, and competitiveness is prohibited from using hormones that are harmful to fish, the environment, and humans who consume this fish. The 17α-MT is one of the hard drugs of the hormone class that is prohibited from being used. Hard drugs are fish medicines which if their use is not in accordance with the provisions can cause danger to fish, environment, and humans.

The Decree of the Ministry of Marine Affairs and Fisheries, Republic of Indonesia (Number:KEP.52/MEN/ 2014) does not set a maximum dose and/or limit of 17α-MT.

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2. Materials and methods

2.1. Test animal

The test animal used was Nile tilapia (*Oreochromis niloticus*). Tilapia fry were produced by artificial fertilization and controlled incubation.

2.2. MT Treatments

MT treatment by oral method was started 3 days after hatching with using 17α-MT (Argent) dose of 60 mg/kg feed. The oral treatment method lasted for 28 days. Immersion method using dose of 0.5 mg/L of 17α-MT was conducted to 10-day-old Tilapia fry and repeated in 13-day-old Tilapia fry for 3 h, respectively [8]. Treatment groups (namely MT-treated fish, both by oral and by immersion) and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment, so the total of fish, both treated and normal were 900 fish.

2.3. Fish rearing

In the initial step, fish were reared at 100 L aquaria, with a density of 1 fish/L for 2 months, separately in each treatment group. Fish was fed on commercial pellet content of 40% crude protein, 3 times daily, at satiation. Sex was determined on 2-month-old fish through manual observation of genitalia for all fish, and gonad histology. To verify the sex, genitalia was obtained from 10 fish/replicate/treatment by using the squash method with acetocarmine dye according to Mukti [8]. Then, male fish of 3 treatments were selected for further study.

In the next step, a total of 360 male tilapias used in this study for 3 treatments (120 fish/treatment) were reared separately at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times. Fish was fed on commercial pellet content of 32% crude protein, 3 times daily, at-satiation.

2.4. Sampling

Commented [xb21cn7]:
Do the dosage of 60 mg/kg and 0.5 mg/L exceed the limit dose? Do they surpass the maximun dose that Minister of Marine Affairs and Fisheries rules?

How to determine the dose of 17α-MT?

Authors response: We was determined the dose of 17α-MT based on several studies that are widely done (according to references) which we have mentioned in this article, one of which is Popma and Green [9].

Commented [xb21cn8]: Why not use 3-day-old fry for immersion as the oral treatment method?

Authors response: Several factors affect the success of sex reversal, including the type and dose of the hormone or material used, the method of treatment, duration of treatment, and age of the fish being treated. Each fish species has a different phase of sex differentiation to be able to receive treatment well and successfully. Therefore, different methods also have difference effects on the different age of fish species.

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Commented [xb21cn9]:
Are the total fish 900?
Actually, you need to state the number of the fish used in the study in the beginning.

Authors response: Yes, we was used a total number of 900 fish larvae or fry, each treatment (oral-treated, immersion-treated, and normal fish) with density of 100 fish and 3 times replication, respectively.
Keep in mind that this treatment was conducted to produce male monosex at laboratory scale before main treatment at grow-out period (field scale) that is the focus of this study.
We have mentioned in the Materials and methods of the article.

Commented [xb21cn10]: You need yo provide a flow chart about the each step of fish rearing. In each step and each treatment, the number of fish should be specified.

Commented [xb21cn11]: This is a basic method and unclear sex result.

Authors response: We have mentioned and added the figure of gonad histology in the Results of the article.

Commented [xb21cn12]: In the "Results" part, The outcome of gonad histology is missing.

Authors response: We have mentioned and added the figure of gonad histology in the Results of the article.

Commented [xb21cn13]:
1. Why did you only use 10 fish for verify the sex in each replicate/treatment? Do the 10 fish represent the full sample?

Commented [xb21cn14]:
Why did you only choose male fish?

If you want to see impact of MT, no need to select only male. Because after MT treatment either oral or immersion, the MT

Commented [xb21cn15]: happa?

Authors response: Happa is net cage

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Commented [xb21cn16]: 4 times?
But, in the last paragraph, treatment was repeated 3 times .

Authors response: Treatment repeated 4 times was used in main treatment at grow-out period (field scale), while treatment repeate

Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively for residue test. 3-month-old fish were used for histology preparation. Fish were anesthetized by using MS222 of 1 mg/L according to Gogal *et al* [165]. Serum (1 mL) was collected according to Atli *et al* [176], and flesh (10 g) of fish was collected to do testing of residues.

2.5. Measurement of MT residue

MT residue, both the serum and the flesh were measured by the sandwich enzyme-linked immunosorbent assay method using fish MT kit (cat number E0103Fi; Bioassay Technology Laboratory, Shanghai, China). Previously, the sample and the reagents were stored at a temperature of 18-25 ^{o}C [3].

2.6. Histology preparation

Fish was carefully dissected on abdominal part according to Wu *et al* [187] and gill, liver, kidneys, ~~and~~ intestine, and gonad organs were collected and stored in the 50 mL tubes which consisted of buffer neutral formalin, with the ratio of 1:2 at room temperature. Histology processes were conducted according to the standard operational procedure, generally with slight modification [198].

2.7. Statistical analysis

Commented [xb21cn17]:
Why is there no gonad histology?

Authors response: We have mentioned preparated gonals histologically in the Materials and methods and added figure in the Results of the article.

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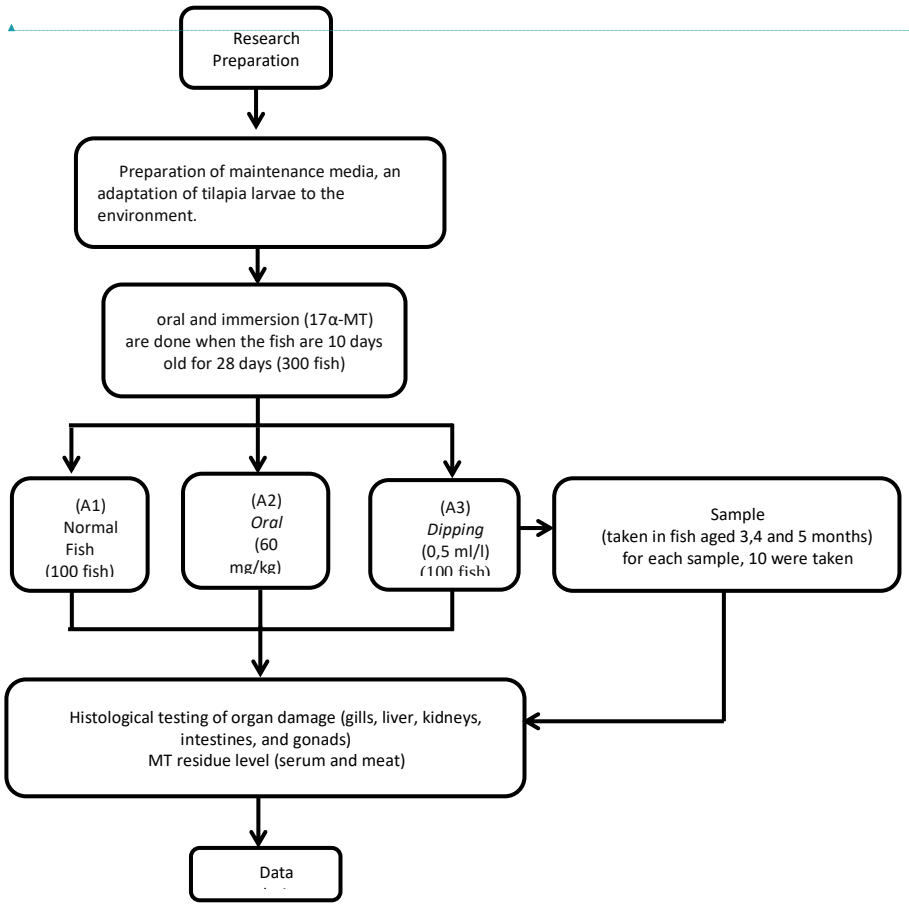
Data of MT residual concentrations were analyzed statistically by using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA was followed by Duncan's multiple range test, while organ histopathology was analyzed descriptively. Data were expressed as mean ± standard deviation (mean ±SD). *P*-value < 0.05 was considered as statistical difference.



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2.8. Ethical approval

The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

3. Results

3.1. MT residual concentrations

MT residual concentration in the serum of MT-treated male tilapia, both by oral and by immersion, was decreased on 4th month while increased again in 5th month, the normal male fish was increased from 3rd month through out the 5th month (Table 1). On 4th and 5th months, the MT residue concentrations were lower in both oral and immersion groups comparing with that of the normal group （*P* both <0.05）.

In the flesh, MT residual concentration showed relatively no significant difference between the oral and immersion treatment groups. MT-treated male tilapia remained lower than normal male tilapia in the 3rd and 4th months, except in the 5th month. However, the result showed that all males had increased MT residue in the 5th month comparing with that of the 4th months(Table 2).

3.2. Organ histopathology

Commented [xb21cn18]: Since the normal fish did not receive MT, why did the MT residue concentrations in normal fish increase with age increasing?

Authors response: We have mentioned in the Discussion of the article

Normal fish have higher MT residue value than MT-treated fish. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

In addition, we have also stated that in this study, we have not been able to test and differentiate the content or residue of MT hormones between the original and those introduced from exogenous (synthetic), so how much is the content or residue of the original MT hormone and the result of introduction, especially in male monosex tilapia from 17α-MT treatment. Therefore, in the future, this limitatation are our concern for further studies.

Commented [xb21cn19]: Why are the values of normal fish higher than the MT-treated fish?

Authors response: We have mentioned in the Discussion of the article.

Normal fish have higher MT residue value than MT-treated fish. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

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MT-treated male tilapias showed histopathology changes in gill, liver, kidneys, and intestine organs (Figure 1). In the gill, such as hyperplasia was found in the bottom secondary lamella. Hypertrophy appeared on the lamella stem due to the occurrence of containment. Clubbing occurred at the end of the primary lamella, which was caused by the existence of retention, so edema appeared on the lamella (Figure 1A). The liver showed congestion, hemorrhage, and cell atrophy (Figure 1B). Congestion was redder due to contained erythrocytes.~~-Hemorrhage was the blood that exit from the centralis.~~ Atrophy was shown by the reduction cell size of Kupper, which made sinusoid widen and made vacuoles degenerate. Congestion caused sinusoidal erythrocytes to wide. Degeneration of liver cells made vacuoles enlarge. Normally, the liver organ did not have damage. Kidneys seem hemorrhage, infiltration of lymphocytes, and neutrophils, inflammation, and necrosis (Figure 1C). The infiltration presence of lymphocytes and neutrophils caused inflammation. The intestine has look atrophy, intestinal villi hemorrhage, lymphoid follicles, and melanomacrophage (Figure 1D). The occurrence of hemorrhage led to the atrophy and melanomacrophage, so finally, it caused erosion and hemorrhage and necrosis of the intestinal villi.

Figure 2 shows an overview of gonadal histology using the squash method with acetocarmine dye. This gonad histology used to differentiate and verify sex of fish in general and easy. On the other hand, testicular histology (Figure 3) used to observe spermatogenesis or testicular development and may be histopathology change in different treatment of 3-month-old fish. This study shows no difference in testicular between normal fish (Figur 3A) and MT-treated fish, both oral (Figure 3B) and immersion (Figure 3C).

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Commented [xb21cn20]: Please add limitation of the study.

Authors response: We have mentioned in the Discussion of the article:

Several limitations of this study are including:

1. unable to measure specificaly the residue of MT which was treated (exogenous) and endogenously by the fish.
2. unable to measure the MT residual concentration in younger fish age.

Therefore, in the future, both of these limitations are our concern for further studies

4. Discussion

Hormonal activities are affected by three stereochemical aspects, *i.e.*, location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration α or β, trans and/or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It is caused by the presence of bacteria in the gastrointestinal tract that oxidizes cluster 17β-hydroxy to be inactive 17β-keto. Therefore, it is necessary to add alkyl group on 17th carbon to become C17α. This prevented the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17α-MT compound has more activity in the body, but it could cause residue. The 17α-MT activity has half the strength of testosterone activity due to the length of C-chain alkyl groups, and then it would decrease androgenic activity. Otherwise, it would increase its toxicity.

The 17α-MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts [20,21~~19,20~~].

Exposure to synthetic chemicals and their residue is risk for human and wildlife health [3,2~~1~~4]. Based on serum MT concentration of males on the 3rd month, orally sex-reversed tilapia had more raising concentration than other treatments. However, in the 4th and 5th months, the MT concentration had decreased every month [3,2~~1~~4]. The orally MT-treated fish would contain MT only in the initial 5 months [2~~3~~3].

~~The testosterone of normal male fish has increased. This was consistent with the study of Khalil *et al*[12] that there was a significant increase in hormone levels in September and October depending on the water temperature and the duration of the dark-light period. This matter caused the beginning of the spawning season of adult fish. It had increased gene expression from steroidogenic enzymes (P450c17, P450scc, and P450arom) to connect the estradiol and testosterone during spawning. The increasing of pheromones indicated it from~~

Commented [xb21cn21]: But in your study, you did not test the testosterone.
Why did you discuss the testosterone?

Authors response: We have deleted these sentences in the Discussion of the article due to no related.

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~~androstenedione 50 ng/h until 1 µg/h. The presence of androstenedione may be caused by an attraction between fish and its opponent sex which improves the setting of reproductive activity.~~

MT concentration was higher in the flesh compared to in the serum. High enough MT concentration was found in the muscle and flesh [24,25~~23,24~~], because the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT to accumulate in the flesh every month. In the research of Pandian and Kirankumar [25~~4~~], exogenous steroid remnants of 5000 ng/kg in fish were too low risk to humans. Endogenous testosterone hormone produced on the testes was 5~~.~~200 ng/kg [26~~5~~], whereas tilapia had endogenous testosterone and estradiol of 3~~.~~000 ng/kg, respectively [25~~4~~].

Normal fish have higher MT residue value than MT-treated fish (Tables 1 and 2). We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

Several limitations of this study are including: a) unable to measure specificaly the MT residual concentration between introduced hormone (exogenous) and endogenously hormone by the fish, and b) unable to measure the MT residual concentration in younger fish age. Therefore, in the future, both of these limitations are our concern for further studies.

Gill layouts that were outside and directly related to water-~~cause the organs~~ would be the first affected by the polluted water environment. The food already digested in the intestines

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Commented [Yan 22]: Please use **ng/kg** instead of ng/g. Please re-calculate the values of hormone.

Authors response: We have revised and changed the unit in the article.

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would be circulated by blood to the liver and kidneys. The liver was the largest organ responsible for metabolism. Kidneys had functioned as a hyperosmotic regulator [276]. In Fish organ took in 3-months-old fish, so at that time, the toxicity to organs is still visibleappeared.

The early stage of damage caused by gill irritation has accompanied the increasing of the mucous cells at the bottom of epithelia with causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached. Gill lamella looked larger than normal which was caused by cell enlargement (hypertrophy), and it looked unclear between the primary and secondary lamellas. According to previous studies [176,287,298], hyperplasia may occur due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination has characterized by a very dense accumulation of red blood cells in the blood vessels, which would block blood vessels (congestion), while edema of lamella looks like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near to the lamella bottom (basal hyperplasia), and then the whole room of interlamellar was filled by new cells which showed like a baseball bat [276,3029].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be returned to normal. Necrosis could not be cured, so if it exposed the tissue activity continuously, then it would decrease cell activity, causing the cells to lose some parts even to death [30,31,32]. Congestion was preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoid widen that accumulated blood and hemorrhage. According to the research of Robert [3029], congestion occurred by the entry of toxic substances into the heart. Hemorrhage was the flow of red blood cells out of the central vein.

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Commented [xb21cn23]: ???
Confusing! Actually, you need to describe the full sentence clearly.

Authors response: We have revised some sentences.

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Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine [32,33,34], which causes a greater concentration of the area mostly to be composed by toxic concentration substances in this area and , causesing damage to the central veinvenous damage. A sinusoid is a small capillary that separated the fundamental of the structural unit with tubule or trabeculae (biliary hepatocytes surrounded by central parenchyma) [32,354]. The liver had enzyme for drug metabolism which is one of the most damaged organs but is very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged. But, it was still capable of regenerating and could even be cured if the damage was lost or destroyed [343].

The infected kidneys were swelling, which was an indication of an inflammatory process that may cause necrosis [354]. Inflammation was an indication of increased lymphocytes and macrophage or neutrophil cell numbers. Kidneys were pollutant-responsive organ to indicate histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach [365]. Changes that often occurred in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression [354-376].

The intestine damage is signed by inflammation. The inflammation or swelling of cells has a reversible characteristic that exposed to the toxic substances in a short period, the cell would return to normal, but if exposed to the toxic substances for a long time, the cell was not able to tolerate damage caused by toxin substances [387]. Melano-macrophage was caused by inflammation which was followed by erosion of the intestinal villi, hemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would disturb the absorption of important substances so that that fish would suffer from malnutrition. In intestinal organs, there were cell swelling, microvillial cell membrane fused,

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Commented [xb21cn24]: Confusing! Actually, you need to describe the full sentence clearly.

Author response: which causes a greater concentration of toxic substances in this area and causes damage to the central vein.

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Authors response: We have revised the word.

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lysis, intestinal vacuum and intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances [210]. Acute intestinal conditions were caused by viruses, parasites, bacteria, algae, and intestinal mucosa. Toxic chemicals could be removed by using mucous epithelial cells that coiled together with the thickening chromatin and cytoplasmic eosinophils [3029]. MT concentrations of serum and flesh have not exceeded the limit (5000 ng/L or 5000 ng/kg) due to the estimated residual synthetic steroid in the fish body of 5000 ng/kg. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histopathological figure of the 4- and 5-month-old fish to determine whether there is a recovery in the fish organ after the cessation of synthetic hormone.

Commented [xb21cn26]: What is the limit of MT concentration?

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Commented [Yan 27]: Please use **ng/kg** instead of ng/g. Please re-calculate the value of steroid.

Authors response: We have revised and changed the unit in the article.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Author contributions

Dewi Nurmalita Suseno, contributes to literature search, clinical and experimental studies, data analysis, and manuscript preparattion.

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Authors response: We have mentioned Author contributions in the article.

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Epy Muhammad Luqman contributes to arrange definition of intellectual content, data analysis, and manuscript review.

Mirni Lamid contributes to arrange definition of intellectual content and statistical analysis.

Akhmad Taufiq Mukti contributes to conceptualization, research design, arrange definition of intellectual content, data acquisition and analysis, and manuscript editing and review.

Muhammad Agus Suprayudi contributes to arrange definition of intellectual content, manuscript review, and guarantor.

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~~[31]~~[32] Sofiana ED, Widjiati, Luqman EM, Samik A. Influence of bark mangosteen *Garcinia mangostana* L. extracts on alveolar type II cell necrosis of the lungs in pregnant neonatal mice *Mus musculus* being cigarette smoke. *J Medik Vet* 2015; **8:** 131-136.

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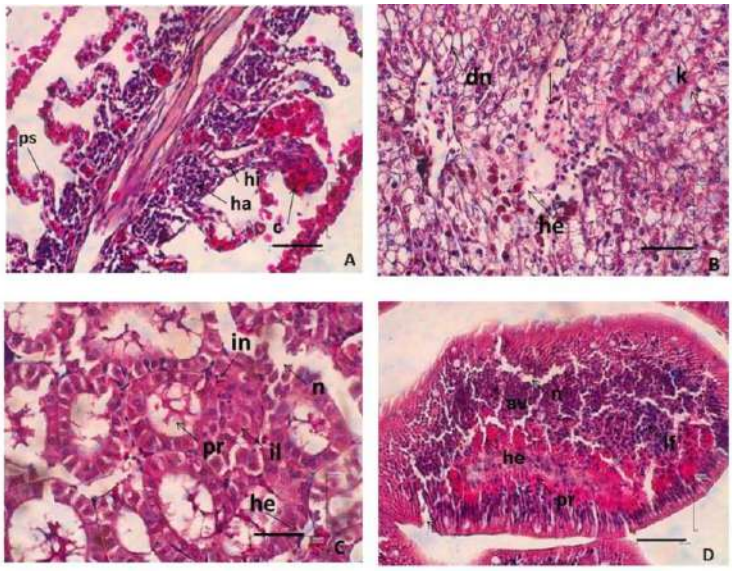

Figure 1. Organ histopathology of 3-month-old male tilapia fish (*n* = 3) (H&E staining, scale bar = 50 µm). (A) gill, (B) liver, (C) kidney, and (D) intestine. Note: ha = hyperplasia; hi = hypertrophy; c = clubbing; ps = bending cell; he = haemorrhage; k = congestion; dn = degeneration of nucleus; il = infiltrating lymphocytes; in = neutrophil infiltration; n = necrosis; pr = inflammation; av = intestinal villi atrophy; lf = lymphoid follicles; pr = inflammation.

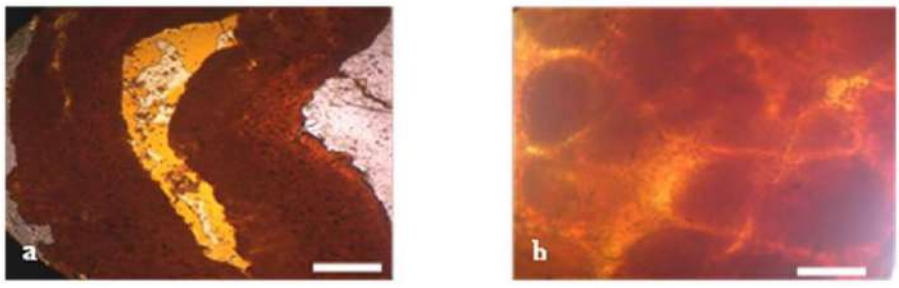

Figure 2 Gonadal histology of male (a= testis) and female (b= ovary) sex tilapias. (Acetocarmine staining; Bar scale = 50 µm)

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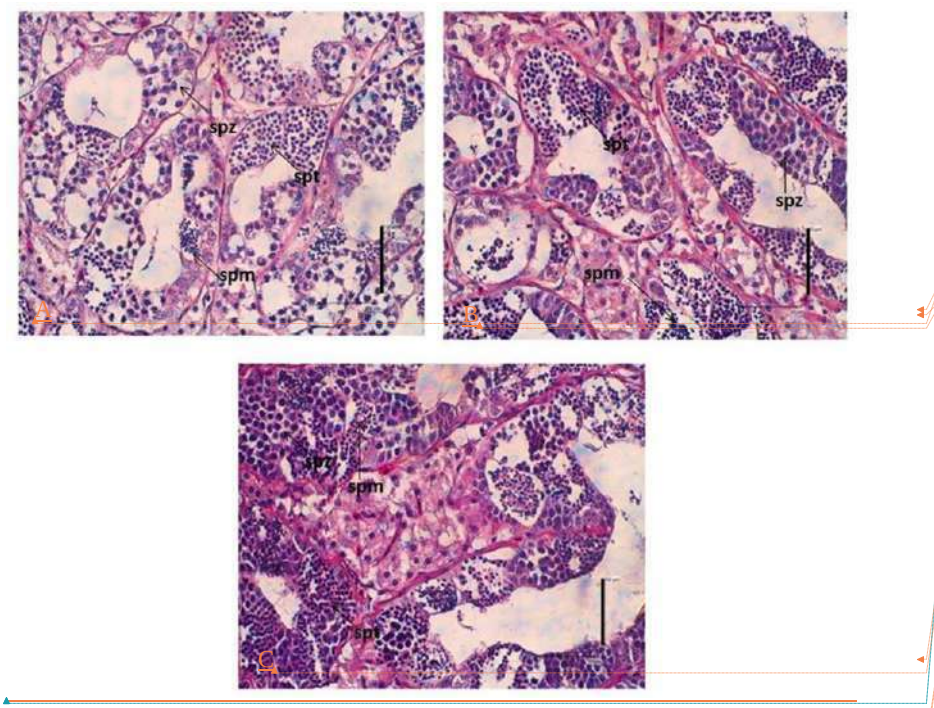

Figure 3 Testicular histology of 3-months-old male tilapia (n = 3): normal fish (A), orally MT-treated fish (B), and MT-immersed fish (C). spm=spermatocytes; spt=spermatid; spz=spermatozoa. (H&E, bar scale = 50 μm).

Table 1. MT residue concentrations (ng/mL) of serum in different age of male tilapia.

Treatments	Ages of tilapia (month)		
	3	4	5
Normal	4.403 ± 0.058 [a]	5.117 ± 0.057 [c]	5.105 ± 0.079 [c]
Oral	5.243 ± 0.080 [b]	4.171 ± 0.051 [b]	4.266 ± 0.050 [a]
Immersion	4.431± 0.029 [a]	3.874 ± 0.038 [a]	4.450 ± 0.054 [b]

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

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Commented [xb21cn36]: Question:

Since the normal fish did not receive MT, why did the MT residue concentrations in normal fish increase with age increasing?

Authors response: We have mentioned in the Discussion of the article.

Normal fish have higher MT residue value than MT-treated fish. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

In addition, we have also stated that in this study, we have not been able to test and differentiate the content or residue of MT hormones between the original and those introduced from exogenous (synthetic), so how much is the content or residue of the original MT hormone and the result of introduction, especially in male monosex tilapia from 17α-MT treatment. Therefore, in the future, this limitatation are our concern for further studies.

Commented [xb21cn37]: Why are the values of normal fish higher than the MT-treated fish?

Authors response: We have mentioned in the Discussion of the article.

Normal fish have higher MT residue value than MT-treated fish. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

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Table 2. MT residue concentrations (ng/g) of the flesh in different age of male tilapia.

Treatments	Ages of tilapia (month)		
	3	4	5
Normal	6.061 ± 0.094 [a]	6.259 ± 0.088 [b]	6.272± 0.109 [a]
Oral	5.967 ± 0.058 [a]	5.995 ± 0.079 [a]	7.099± 0.135 [b]
Immersion	5.900 ± 0.100 [a]	5.898 ± 0.079 [a]	6.403 ± 0.088 [a]

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

Commented [xb21cn38]: Since the normal fish did not receive MT, why did the MT residue concentrations in normal fish increase with age increasing?

Authors response: We have mentioned in the Discussion of the article.

Normal fish have higher MT residue value than MT-treated fish. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

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Dear Miss. Suseno
Asian Pacific Journal of Reproduction has received your revised manuscript entitled '"Residual impact of 17a-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (Oreochromis niloticus)"..' The manuscript will be re-evaluated by concerned referees for the final decision regarding its suitability for publication. We will get back to you within four weeks.
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Thank you for revised manuscript.

The revised manuscript has been sent to assessment.

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Before we typset your manuscript, please address the following issues:

1. The picture of the Figure 3 seems the reference substance not the results of your study. Please check! Actually, you need to add Figure note illustrating the Figure's detailed content.

2. Fish should prove to be male. If you are not able to prove, you may replace Figure 3 with word descriptions in the text (Delete Figure 3). And make the results of gonadal histology sense and justified.
Anyway, please justify this part. Thank you!

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3. Please proofread the full text.

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Dear authors, before we typset your manuscript, please address the following issues:

1. The picture of the Figure 3 seems the reference substance **not** the results of your study. Please check! Actually, you need to add **Figure note** illustrating the Figure's detailed content.

Authors response: The Figure 3 is the result of a study conducted by Mukti [8], as we have mentioned in the Materials and methods, Results, and Figure of the article and their illustration.

2. Fish should **prove** to be male. If you are not able to prove, you may replace Figure 3 with word descriptions in the text (Delete Figure 3). And make the results of gonadal histology sense and **justified.** Anyway, please justify this part. Thank you!

Authors response: In this study, we are convinced that we only use male fish, as we have mentioned in the Materials and methods of the article. Figure 3 is used to show the differences in male and female gonad preparations as evidence in verify the sex from genetalia observation.

PS: If you keep Figure 3, please provide the picture of higher resolution (dpi>300) by email (apjr2012@163.com).

3. Please proofread the full text.

Authors response: We have proofread the full text. We have corrected and mentioned some sentences (colored blue) in the text of the article.

All revisions should be maked in color. Thank you!

_Editor Lin

2019.12.20

Residual impact of 17α**-methyltestosterone and histopathological changes in sex-reversed *Oreochromis niloticus***

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Abstract

Objective: To examine sex reversal both by oral and by immersion using 17α-methyltestosterone on the methyltestosterone residual concentration and the organ histopathology of tilapia fish.

Methods: This study used oral and immersion treatment methods for sex reversal of tilapia fish and used normal fish as control and each treatment was repeated 4 times. Dosages of 17α-methyltestosterone 60 mg/kg feed and 0.5 mL/L of 17α-methyltestosterone were used for oral and immersion methods, respectively. In the first step, tilapia fry were reared at 100 L aquaria, with a density of 1 fish/L for 2 months. In the next step, male tilapias were reared at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with a density of 30 fish/happa for 3 months. The methyltestosterone residual concentrations were analyzed by one-way analysis of variance and Duncan's multiple range tests, while organ histopathology was analyzed by descriptive method.

Results: Residual concentrations in the serum of methyltestosterone-treated fish were significnat lower than that in normal fish, especially in 4- and 5-month-old tilapias with averages of less than 5 mg/L, while in normal fish was more than 5 mg/L. In the flesh, methyltestosterone residual concentrations showed relatively no significant differences between oral and immersion treatment groups and methyltestosterone-treated fish remains lower compared to normal fish, except in 5-month-old tilapia. Methyltestosterone-treated tilapia have indicated histopathological changes on gill, liver, kidneys, and intestine organs.

Conclusions: Sex reversal either by oral or by immersion has methyltestosterone residual concentration did not exceed the limits (5 mg/L or 5 mg/kg) of synthetic steroid on the fish body, although methyltestosterone causes histopathological changes on gill, liver, kidneys, and intestine organs.

Keywords:

17α-methyltestosterone

Residue

Organ histopathology

Tilapia

Sex reversal method

1. Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be a simple, easy, and highly effective technology [1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT) [2,3] is a derivative of testosterone[4], which potentially increases sexual developmental in males [3]. The 17α-MT-immersed tilapia larvae produce males of 91.6%-98.3% [5,6], however, oral treatment of 60 mg/kg feed produces males of 93.7% [7], 97.7% [8], even reaches up to 100% males [9].

Synthetic steroid hormone would enter through the blood vessels in the body and then it was modulated by the brain and pituitary hormones [10]. Steroid hormone was synthesized in either the liver or the kidneys [11], and subsequently, it would produce androstenedione which consists of 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sex, but 17α-MT has characteristic that it is difficult to be absorbed within the body and it will also contaminate environment [12].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (a carcinogenic and endocrine disorder) as well as the danger to environment [1,3,13-15]. The group of anabolic steroids (including 17α-MT) based on the decision of Ministry of Marine Affairs and Fisheries, Republic of Indonesia (number KEP.52/MEN/2014) has been banned because the hormones were harmful to fish, environment, and human. This study expected to prove the presumption that has been the subject of debate in the fish farmer community that the use of 17α-MT at any dose produces dangerous and toxic residues when consumed by humans and the released into the environment, as well as the debate among researchers and to address concerns that have existed in the community and policy makers that the use of 17α-MT in certain doses is still safe and does not contain dangerous residues of concern so far. So that the regulation can be revised again for the advancement of aquaculture while maintaining a sustainable environment and human health that consumes cultured fish. Therefore, the aims of the study were to examine sex reversal both by oral and by immersion using 17α-MT on the MT residual concentration and the organ histopathology changes of tilapia.

2. Materials and methods

2.1. Test animal

The test animal used was Nile tilapia (*Oreochromis niloticus*). Tilapia fry were produced by artificial fertilization and controlled incubation.

2.2. MT treatments

MT treatment by oral method was started 3 days after hatching with using 17α-MT (Argent) dose of 60 mg/kg feed. The oral treatment method lasted for 28 days. Immersion method using dose of 0.5 mg/L of 17α-MT was conducted to 10-day-old Tilapia fry and repeated in 13-day-old Tilapia fry for 3 h, respectively [8]. Treatment groups (namely MT-treated fish, both by oral and by immersion) and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment, so the total of fish, both treated and normal were 900 fish.

2.3. Fish rearing

In the initial step, fish were reared at 100 L aquaria, with a density of 1 fish/L for 2 months, separately in each treatment group. Fish was fed on commercial pellet content of 40% crude protein, 3 times daily, at satiation. Sex was determined on 2-month-old fish through manual observation of genitalia for all fish, and gonad preparation~~histology~~. To verify the sex from genitalia observation, gonad ~~genitalia~~ was obtained from 10 fish/replicate/treatment by using the squash method with acetocarmine dye according to Mukti [8]. Based on 17α-MT hormone treatment either by oral or by immersion and verify the sex by fish genetalia observation and followed by gonad preparation shows male of 97-98% and female of 2-3% [8]. Then, male fish of 3 treatments were selected for further study.

In the next step, a total of 360 male tilapias used in this study for 3 treatments (120 fish/treatment) were reared separately at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times. Fish was fed on commercial pellet content of 32% crude protein, 3 times daily, at-satiation.

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2.4. Sampling

Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively for residue test. 3-month-old fish were used for histology preparation. Fish were anesthetized by using MS222 of 1 mg/L according to Gogal *et al* [16]. Serum (1 mL) was collected according to Atli *et al* [17], and flesh (10 g) of fish was collected to do testing of residues.

2.5. Measurement of MT residue

MT residue, both the serum and the flesh were measured by the sandwich enzyme-linked immunosorbent assay method using fish MT kit (cat number E0103Fi; Bioassay Technology Laboratory, Shanghai, China). Previously, the sample and the reagents were stored at a temperature of 18-25 ^{o}C [3].

2.6. Histology preparation

Fish was carefully dissected on abdominal part according to Wu *et al* [18] and gill, liver, kidneys, intestine, and gonad organs were collected and stored in the 50 mL tubes which consisted of buffer neutral formalin, with the ratio of 1:2 at room temperature. Histology processes were conducted according to the standard operational procedure, generally with slight modification [19].

2.7. Statistical analysis

Data of MT residual concentrations were analyzed statistically by using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA was followed by Duncan's multiple range test, while organ histopathology was analyzed descriptively. Data were expressed as mean ± standard deviation (mean ±SD). *P*-value < 0.05 was considered as statistical difference.

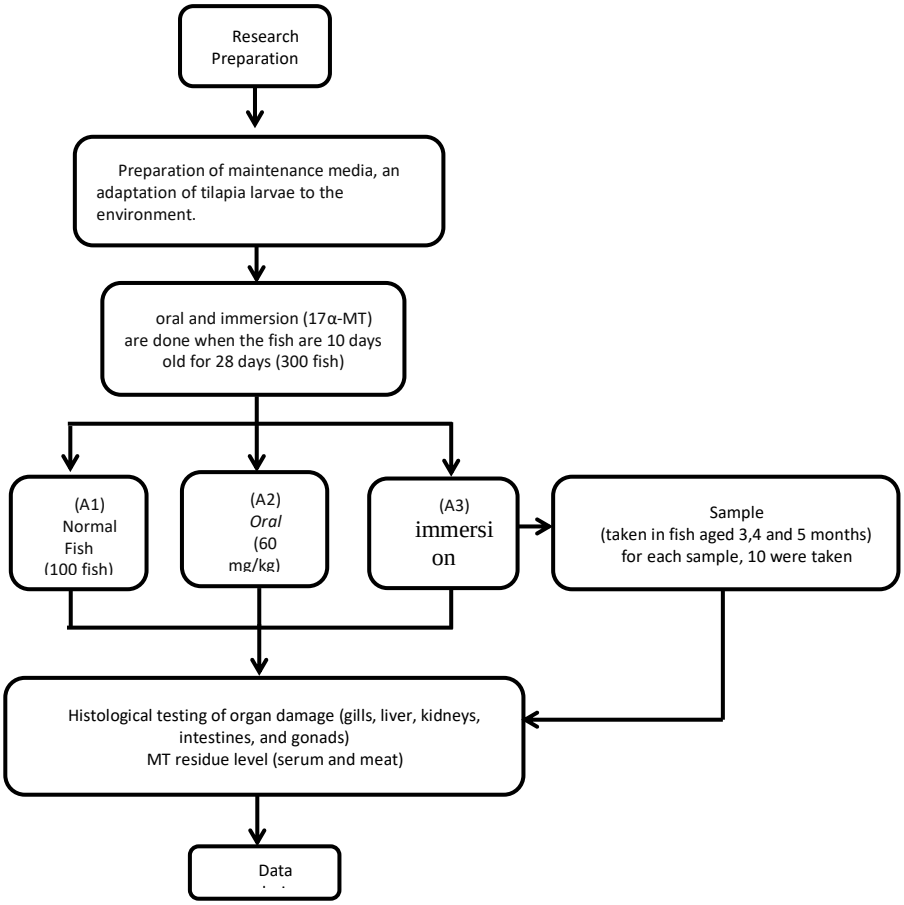

Figure 1. Flow chart of the study.

2.8. Ethical approval

The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

3. Results

3.1. MT residual concentrations

MT residual concentration in the serum of MT-treated male tilapia, both by oral and by immersion, was decreased on 4th month while increased again in 5th month, the normal male fish was increased from 4th month while slightly decreased in 5th month (Table 1). On 4th and 5th months, the MT residue concentrations were lower in both oral and immersion groups comparing with that of the normal group（*P* both <0.05）.

In the flesh, MT residual concentration showed relatively no significant difference between the oral and immersion treatment groups on 4th month, but the MT residual was significant higher in oral treatment group than that in the immersion group. MT-treated male tilapia remained lower than normal male tilapia in the 3rd and 4th months, except in the 5th month. However, the result showed that all males had increased MT residue in the 5th month comparing with that of the 4th months (Table 2).

3.2. Organ histopathology

MT-treated male tilapias showed histopathology changes in gill, liver, kidneys, and intestine organs (Figure 2). In the gill, such as hyperplasia was found in the bottom secondary lamella. Hypertrophy appeared on the lamella stem due to the occurrence of containment. Clubbing occurred at the end of the primary lamella, which was caused by the existence of retention, so edema appeared on the lamella (Figure 2A). The liver showed congestion, hemorrhage, and cell atrophy (Figure 2B). Congestion was redder due to contained erythrocytes.- Atrophy was shown by the reduction cell size of Kupper, which made sinusoid widen and made vacuoles degenerate. Congestion caused sinusoidal erythrocytes to wide. Degeneration of liver cells made vacuoles enlarge. Normally, the liver organ did not have damage. Kidneys seem hemorrhage, infiltration of lymphocytes, and neutrophils, inflammation, and necrosis (Figure 2C). The infiltration presence of lymphocytes and neutrophils caused inflammation. The intestine has look atrophy, intestinal villi hemorrhage, lymphoid follicles, and melanomacrophage (Figure 2D). The occurrence of hemorrhage led to the atrophy and melanomacrophage, so finally, it caused erosion and hemorrhage and necrosis of the intestinal villi.

Figure 3 [8] showed an overview of gonad preparational ~~histology~~ using the squash method with acetocarmine dye. This gonad preparation~~histology~~ was used to differentiate and verify sex of fish in general and easy. Male gonad of fish (testis) indicate spermatocyte form (3A), while female gonad of fish (ovary) show oocyte form (3B) in the gonad preparation. On the other hand, testicular histology (Figure 4) used to observe spermatogenesis or testicular development and may be histopathology change in different treatment of 3-month-old fish. This study showed no difference in testicular between normal fish (Figur 4A) and MT-treated fish, both oral (Figure 4B) and immersion (Figure 4C).

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1. Dear authors, the picture of the Figure 3 seems the reference substance **not** the results of your study. Please check! Actually, you need to add **Figure note** illustrating the Figure's detailed content.

Authors response: The Figure 3 is the result of a study conducted by Mukti [8], as we have mentioned in the Materials and methods, Results, and Figure of the article and their illustration

2. Fish should **prove** to be male. If you are not able to prove, you may replace Figure 3 with word descriptions in the text (Delete Figure 3). And make the results of gonadal histology sense and **justified.**
Anyway, please justify this part. Thank you!

Authors response: In this study, we are convinced that we only use male fish, as we have mentioned in the Materials and methods of the article. Figure 3 is used to show the differences in male and female gonad preparations as evidence in verify the sex from genetalia observation

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4. Discussion

Hormonal activities are affected by three stereochemical aspects, *i.e.*, location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration α or β, trans and/or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It is caused by the presence of bacteria in the gastrointestinal tract that oxidizes cluster 17β-hydroxy to be inactive 17β-keto. Therefore, it is necessary to add alkyl group on 17th carbon to become C17α. This prevented the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17α-MT compound has more activity in the body, but it could cause residue. The 17α-MT activity has half the strength of testosterone activity due to the length of C-chain alkyl groups, and then it would decrease androgenic activity. Otherwise, it would increase its toxicity.

The 17α-MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts [20,21].

Exposure to synthetic chemicals and their residue is risk for human and wildlife health [3,22]. Based on serum MT concentration of males on the 3rd month, orally sex-reversed tilapia had more raising concentration than other treatments. However, in the 4th and 5th months, the MT concentration had decreased every month [3,22]. The orally MT-treated fish would contain MT only in the initial 5 months [23].

~~The testosterone of normal male fish has increased. This was consistent with the study of Khalil *et al*[12] that there was a significant increase in hormone levels in September and~~

~~October depending on the water temperature and the duration of the dark-light period. This matter caused the beginning of the spawning season of adult fish. It had increased gene expression from steroidogenic enzymes (P450c17, P450scc, and P450arom) to connect the estradiol and testosterone during spawning. The increasing of pheromones indicated it from androstenedione 50 ng/h until 1 µg/h. The presence of androstenedione may be caused by an attraction between fish and its opponent sex which improves the setting of reproductive activity.~~

MT concentration was higher in the flesh compared to in the serum. High enough MT concentration was found in the muscle and flesh [24,25], because the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT to accumulate in the flesh every month. In the research of Pandian and Kirankumar [25], exogenous steroid remnants of 5000 ng/kg in fish were too low risk to humans. Endogenous testosterone hormone produced on the testes was 5200 ng/kg [26], whereas tilapia had endogenous testosterone and estradiol of 3000 ng/kg, respectively [25]. Normal fish have higher MT residue value than MT-treated fish as shown in this study. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

Several limitations of this study are including: a) unable to measure specificaly the MT residual concentration between introduced hormone (exogenous) and endogenously hormone

by the fish, and b) unable to measure the MT residual concentration in younger fish age. Therefore, in the future, both of these limitations are our concern for further studies.

Gill layouts that were outside and directly related to water-would be the first affected by the polluted water environment. The food already digested in the intestines would be circulated by blood to the liver and kidneys. The liver was the largest organ responsible for metabolism. Kidneys had functioned as a hyperosmotic regulator [27]. In 3-months-old fish, , the toxicity to organs is still visible.

The early stage of damage caused by gill irritation has accompanied the increasing of the mucous cells at the bottom of epithelia with causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached. Gill lamella looked larger than normal which was caused by cell enlargement (hypertrophy), and it looked unclear between the primary and secondary lamellas. According to previous studies [17,28,29], hyperplasia may occur due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination has characterized by a very dense accumulation of red blood cells in the blood vessels, which would block blood vessels (congestion), while edema of lamella looks like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near to the lamella bottom (basal hyperplasia), and then the whole room of interlamellar was filled by new cells which showed like a baseball bat [27,30].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be returned to normal. Necrosis could not be cured, so if it exposed the tissue activity continuously, then it would decrease cell activity, causing the cells to lose some parts even to death [31,32]. Congestion was preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoid widen that accumulated blood and

hemorrhage. According to the research of Robert [30], congestion occurred by the entry of toxic substances into the heart. Hemorrhage was the flow of red blood cells out of the central vein.

Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine [33,34], which causes a greater concentration of toxic substances in this area and causes damage to the central vein. A sinusoid is a small capillary that separated the fundamental of the structural unit with tubule or trabeculae (biliary hepatocytes surrounded by central parenchyma) [33,35]. The liver had enzyme for drug metabolism which is one of the most damaged organs but is very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged. But, it was still capable of regenerating and could even be cured if the damage was lost or destroyed [34].

The infected kidneys were swelling, which was an indication of an inflammatory process that may cause necrosis [35]. Inflammation was an indication of increased lymphocytes and macrophage or neutrophil cell numbers. Kidneys were pollutant-responsive organ to indicate histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach [36]. Changes that often occurred in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression [35-37].

The intestine damage is signed by inflammation. The inflammation or swelling of cells has a reversible characteristic that exposed to the toxic substances in a short period, the cell would return to normal, but if exposed to the toxic substances for a long time, the cell was not able to tolerate damage caused by toxin substances [38]. Melano-macrophage was caused by inflammation which was followed by erosion of the intestinal villi, hemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would

disturb the absorption of important substances so that that fish would suffer from malnutrition. In intestinal organs, there were cell swelling, microvillicell membrane fused, lysis, intestinal vacuum and intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances [21]. Acute intestinal conditions were caused by viruses, parasites, bacteria, algae, and intestinal mucosa. Toxic chemicals could be removed by using mucous epithelial cells that coiled together with the thickening chromatin and cytoplasmic eosinophils [30]. MT concentrations of serum and flesh have not exceeded the limit (5000 ng/L or 5000 ng/kg) due to the estimated residual synthetic steroid in the fish body of 5000 ng/kg. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histopathological figure of the 4- and 5-month-old fish to determine whether there is a recovery in the fish organ after the cessation of synthetic hormone.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Author contributions

Dewi Nurmalita Suseno contributes to literature search, clinical and experimental studies, data analysis, and manuscript preparattion. Epy Muhammad Luqman contributes to arrange

definition of intellectual content, data analysis, and manuscript review. Mirni Lamid contributes to arrange definition of intellectual content and statistical analysis. Akhmad Taufiq Mukti contributes to conceptualization, research design, arrange definition of intellectual content, data acquisition and analysis, and manuscript editing and review. Muhammad Agus Suprayudi contributes to arrange definition of intellectual content, manuscript review, and guarantor.

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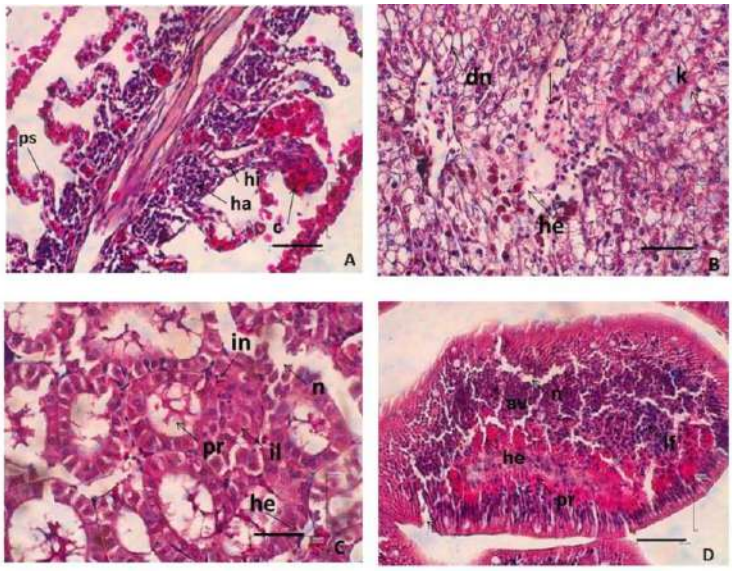
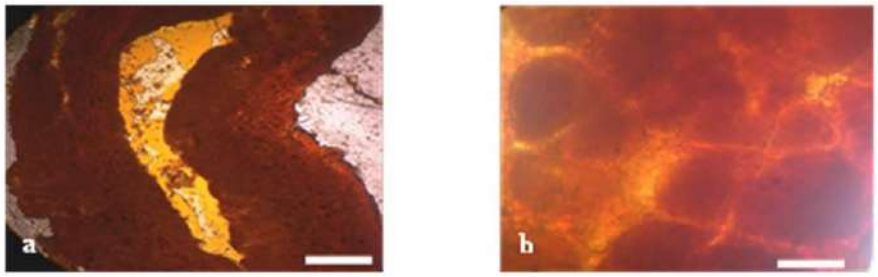


Figure 2. Organ histopathology of 3-month-old male tilapia fish (*n* = 3) (H&E staining, scale bar = 50 μm). (A) gill, (B) liver, (C) kidney, and (D) intestine. Note: ha = hyperplasia; hi = hypertrophy; c = clubbing; ps = bending cell; he = haemorrhage; k = congestion; dn = degeneration of nucleus; il = infiltrating lymphocytes; in = neutrophil infiltration; n = necrosis; pr = inflammation; av = intestinal villi atrophy; lf = lymphoid follicles; pr = inflammation.



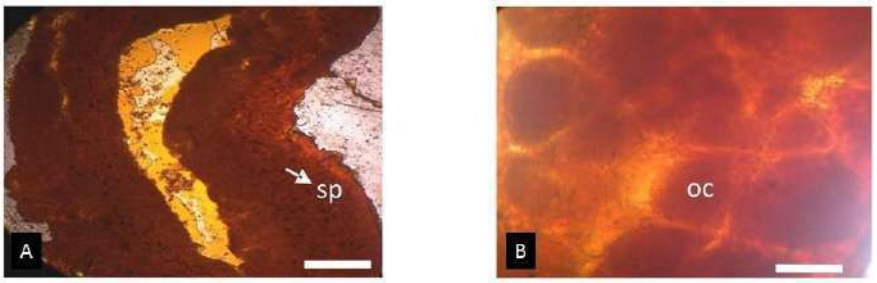


Figure 32 Gonad preparational histology of male (Aa= testis) and female (Bb= ovary) sex tilapias, sp = spermatocyte, oc = oocyte, (Acetocarmine staining; Bar scale = 50 µm) [8]

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1. Dear authors, the picture of the Figure 3 seems the reference substance **not** the results of your study. Please check!
Actually, you need to add **Figure note** illustrating the Figure's detailed content.

2. Fish should **prove** to be male. If you are not able to prove, you may replace Figure 3 with word descriptions in the text (Delete Figure 3). And make the results of gonadal histology sense and **justified.**
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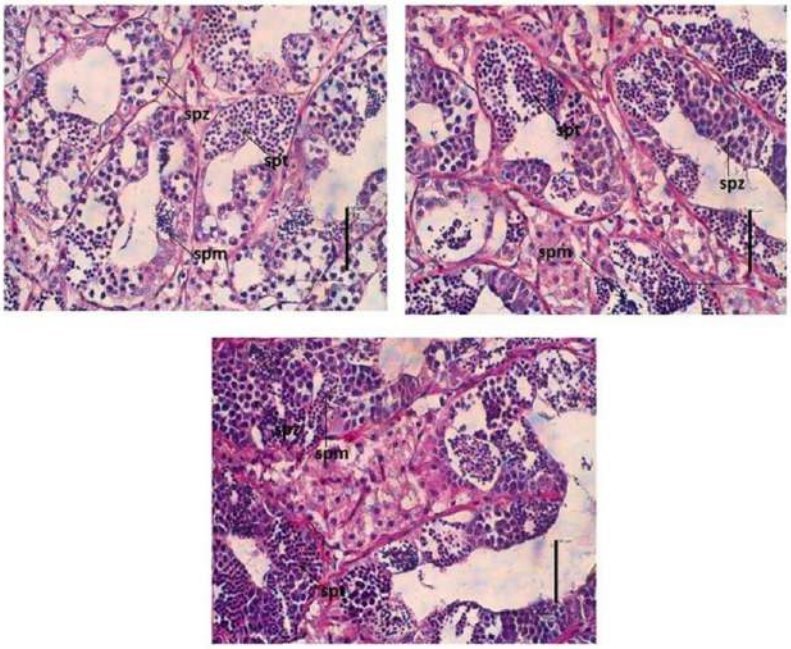

Figure 4. Testicular histology of 3-month-old male tilapia (n = 3); normal fish (A), orally MT-treated fish (B), and MT-immersed fish (C), spm=spermatocytes; spt=spermatid; spz=spermatozoa. (H&E, bar scale = 50 μm).

Table 1. MT residual concentrations (mg/mL) of serum in different age of male tilapia.

Treatments	Ages of tilapia (month)		
	3	4	5
Normal	4.403 ± 0.058 [a]	5.117 ± 0.057 [c]	5.105 ± 0.079 [c]
Oral	5.243 ± 0.080 [b]	4.171 ± 0.051 [b]	4.266 ± 0.050 [a]
Immersion	4.431± 0.029 [a]	3.874 ± 0.038 [a]	4.450 ± 0.054 [b]

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

Table 2. MT residual concentrations (mg/g) of the flesh in different age of male tilapia.

Treatments	Ages of tilapia (month)		
	3	4	5
Normal	6.061 ± 0.094 [a]	6.259 ± 0.088 [b]	6.272± 0.109 [a]
Oral	5.967 ± 0.058 [a]	5.995 ± 0.079 [a]	7.099± 0.135 [b]
Immersion	5.900 ± 0.100 [a]	5.898 ± 0.079 [a]	6.403 ± 0.088 [a]

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

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1	The picture of the Figure 3 seems the reference substance **not** the results of your study. Please check! Actually, you need to add Figure note illustrating the Figure's detailed content	The Figure 3 is the result of a study conducted by Mukti [8], as we have mentioned in the Materials and methods, Results, and Figure of the article and their illustration	
2	Fish should **prove** to be male. If you are not able to prove, you may replace Figure 3 with word descriptions in the text (Delete Figure 3). And make the results of gonadal histology sense and **justified.** Anyway, please justify this part. Thank you!	In this study, we are convinced that we only use male fish, as we have mentioned in the Materials and methods of the article. Figure 3 is used to show the differences in male and female gonad preparations as evidence in verify the sex from genetalia observation.	
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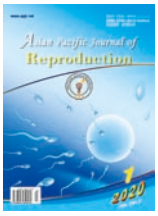

Original Article

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Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Objective: To examine sex reversal both by oral and by immersion using 17α-methyltestosterone on the methyltestosterone residual concentration and the organ histopathology of tilapia fish.

Methods: This study used oral and immersion treatment methods for sex reversal of tilapia fish and used normal fish as the control and each treatment was repeated 4 times. 17α-methyltestosterone at dosages of 60 mg/kg feed and 0.5 mg/L were used for oral and immersion methods, respectively. In the first step, tilapia fry were reared at 100 L aquaria, with a density of 1 fish/L for 2 months. In the next step, male tilapias were reared at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with a density of 30 fish/happa for 3 months. The methyltestosterone residual concentrations were analyzed by one-way analysis of variance and Duncan's multiple range tests, while organ histopathology was analyzed by descriptive method.

Results: Residual concentrations in the serum of methyltestosterone-treated fish were significantly lower than that in normal fish, especially in 4- and 5-month-old tilapias with averages of less than 5 μg/L, while in normal fish was more than 5 μg/L. In the flesh, methyltestosterone residual concentrations showed relatively no significant differences between the oral and immersion treatment groups and methyltestosterone-treated fish remained lower compared to normal fish, except in 5-month-old tilapia. Methyltestosterone-treated tilapia exhibited histopathological changes on gill, liver, kidneys, and intestine organs.

Conclusions: Sex reversal either by oral or by immersion has methyltestosterone residual concentration, but does not exceed the limits (5 μg/L or 5 μg/kg) of synthetic steroid on the fish body, although methyltestosterone causes histopathological changes on gill, liver, kidneys, and intestine.

KEYWORDS: 17 α -methyltestosterone; Residue; Organ histopathology; Tilapia; Sex reversal method

1. Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be a simple, easy, and highly effective technology[1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT)[2,3] is a derivative of testosterone[4], which potentially increases sexual developmental in males[3]. The 17α-MT-immersed tilapia larvae produce males of 91.6%-98.3% [5,6], however, oral treatment of 60 mg/kg feed produces males of 93.7%[7], 97.7%[8], even reaches up to 100% males[9].

Synthetic steroid hormone would enter through the blood vessels in the body and then it was modulated by the brain and pituitary hormones[10]. Steroid hormone was synthesized in either the liver or the kidneys[11], and subsequently, it would produce androstenedione which consists of 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sex, but 17α-MT has characteristics that it is difficult to be absorbed within the body and it will also contaminate the environment[12].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (a carcinogenic and endocrine disorder) as well as the danger to the environment[1,3,13-15]. The group of anabolic

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steroids (including 17α-MT) based on the decision of the Ministry of Marine Affairs and Fisheries, Republic of Indonesia (number KEP.52/MEN/2014) has been banned because of the hormones harmful to fish, environment, and human. This study expect to prove the presumption that has been the subject of debate in the fish farmer community that the use of 17α-MT at any dosage produces dangerous and toxic residues when consumed by humans and the released into the environment, as well as the debate among researchers and to address concerns that have existed in the community and policymakers that the use of 17α-MT in certain doses is still safe and does not contain dangerous residues of concern so far. So that the regulation can be revised again for the advancement of aquaculture while maintaining a sustainable environment and human health that consumes cultured fish. Therefore, the study aimed to examine sex reversal both by oral and by immersion using 17α-MT on the MT residual concentration and the organ histopathology changes of tilapia.

2. Materials and methods

2.1. Test animal

The test animal used was Nile tilapia (*Oreochromis niloticus*). Tilapia fry were produced by artificial fertilization and controlled incubation.

2.2. MT treatments

MT treatment by the oral method was started 3 days after hatching with using 17α-MT (Argent) dosage of 60 mg/kg feed. The oral treatment method lasted for 28 days. Immersion method using dosage of 0.5 mg/L of 17α-MT was conducted to 10-day-old Tilapia fry and repeated in 13-day-old Tilapia fry for 3 h, respectively[8]. Treatment groups (namely MT-treated fish, both by oral and by immersion) and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment, so the total of fish, both treated and normal were 900 fish.

2.3. Fish rearing

In the initial step, fish were reared at 100 L aquaria, with a density of 1 fish/L for 2 months, separately in each treatment group. Fish was fed on commercial pellet content of 40% crude protein, 3 times daily, at satiation. Sex was determined on 2-month-old fish through manual observation of genitalia for all fish, and gonad preparation. To verify the sex from genitalia observation, gonad was obtained from 10 fish/replicate/treatment by using the squash method with acetocarmine dye according to Mukti[8]. Based on 17α-MT hormone treatment either by oral or by immersion and verify the sex by fish genetalia observation and followed by gonad preparation shows male of 97%-98% and female of 2%-3%[8]. Then, male fish of 3 treatments were selected for further study.

In the next step, a total of 360 male tilapias used in this study for 3 treatments (120 fish/treatment) were reared separately at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times. Fish was fed on commercial pellet content of 32% crude protein, 3 times daily, at-satiation.

2.4. Sampling

Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively for residue test. 3-month-old fish were used for histology preparation. Fish were anesthetized by using MS222 of 1 mg/L according to Gogal *et al*[16]. Serum (1 mL) was collected according to Atli *et al*[17], and flesh (10 g) of fish was collected to do testing of residues.

2.5. Measurement of MT residue

MT residue, both the serum and the flesh were measured by the sandwich enzyme-linked immunosorbent assay method using fish MT kit (cat number E0103Fi; Bioassay Technology Laboratory, Shanghai, China). Previously, the sample and the reagents were stored at a temperature of 18 ℃-25 ℃[3].

2.6. Histology preparation

Fish was carefully dissected on the abdominal part according to Wu *et al*[18] and gill, liver, kidneys, intestine, and gonad organs were collected and stored in the 50 mL tubes which consisted of buffer neutral formalin, with the ratio of 1:2 at room temperature. Histology processes were conducted according to the standard operational procedure, generally with slight modification[19]. The flow chart of the study was shown in Figure 1.

2.7. Statistical analysis

Data of MT residual concentrations were analyzed statistically by using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA was followed by Duncan's multiple range test, while organ histopathology was analyzed descriptively. Data were expressed as mean ± standard deviation (mean ± SD). *P*–value < 0.05 was considered as statistical difference.

2.8. Ethical approval

The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

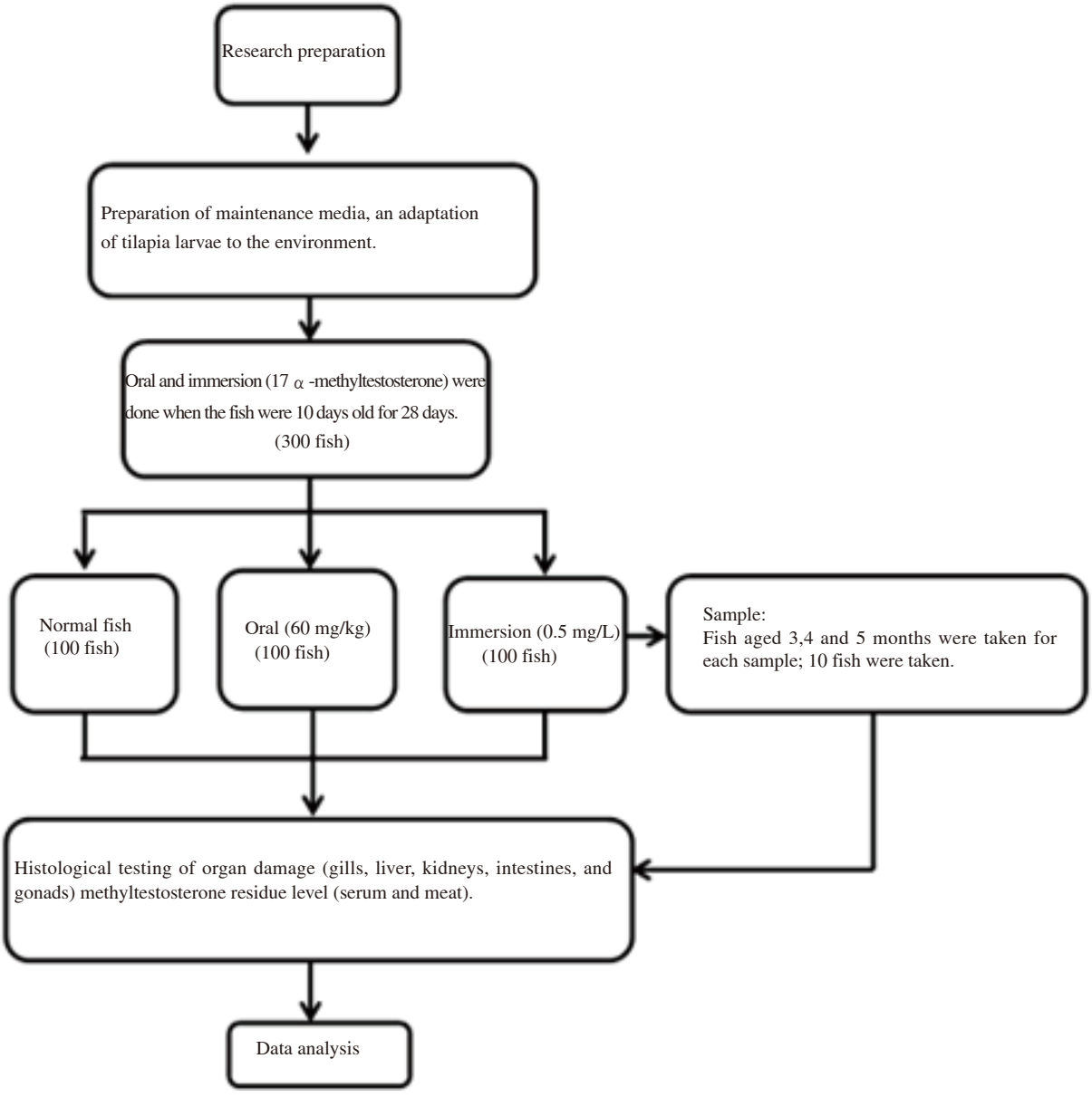

Figure 1. Flow chart of the study.

3. Results

3.1. MT residual concentrations

MT residual concentration in the serum of MT-treated male tilapia, both by oral and by immersion, was decreased on the 4th month while increased again on the 5th month, the normal male fish was increased from the 4th month while slightly decreased on the 5th month (Table 1). On the 4th and 5th months, the MT residue concentrations were lower in both oral and immersion groups comparing with that of the normal group (P both <0.05).

In the flesh, MT residual concentration showed relatively no significant difference between the oral and immersion treatment groups on 4th month, but the MT residual was significantly higher in the oral treatment group than that in the immersion group. MT-treated male tilapia remained lower than normal male tilapia in the 3rd and 4th months, except in the 5th month. However, the result showed that all males had increased MT residue in the 5th month comparing with that of the 4th month (Table 2).

3.2. Organ histopathology

MT-treated male tilapias showed histopathology changes in gill, liver, kidneys, and intestine organs (Figure 2). In the gill, such as hyperplasia was found in the bottom secondary lamella. Hypertrophy appeared on the lamella stem due to the occurrence of containment. Clubbing occurred at the end of the primary lamella, which was caused by the existence of retention, so edema appeared on the lamella (Figure 2A). The liver showed congestion, hemorrhage, and cell atrophy (Figure 2B). Congestion was redder due to contained erythrocytes. Atrophy was shown by the reduction of cell size of Kupper, which made sinusoid widen and made vacuoles degenerate. Congestion caused sinusoidal erythrocytes to wide. Degeneration of liver cells made vacuoles enlarge. Normally, the liver organ did not have damage. Kidneys seem hemorrhage, infiltration of lymphocytes, and neutrophils, inflammation, and necrosis (Figure 2C). The infiltration presence of lymphocytes and neutrophils caused inflammation. The

intestine has look atrophy, intestinal villi hemorrhage, lymphoid follicles, and melanomacrophage (Figure 2D). The occurrence of hemorrhage led to the atrophy and melanomacrophage, so finally, it caused erosion and hemorrhage and necrosis of the intestinal villi. On the other hand, testicular histology (Figure 3) used to observe spermatogenesis or testicular development and may be histopathology change in different treatment of 3-month-old fish. This study showed no difference in testicular between normal fish (Figur 3A) and MT-treated fish, both oral (Figure 3B) and immersion (Figure 3C).

Table 1. Methyltestosterone residual concentrations (μg/L) of serum in different age of male tilapia.

Ages of tilapia (month)	Treatments		
	Normal	Oral	Immersion
3	4.403 ± 0.058^{8a}	5.243 ± 0.080^{b}	4.431 ± 0.029^{a}
4	5.117 ± 0.057^{c}	4.171 ± 0.051^{b}	3.874 ± 0.038^{a}
5	5.105 ± 0.079^{c}	4.266 ± 0.050^{a}	4.450 ± 0.054^{b}

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

Table 2. Methyltestosterone residual concentrations (μg/kg) of the flesh in different age of male tilapia.

Ages of tilapia (month)	Treatments		
	Normal	Oral	Immersion
3	6.061 ± 0.094^{a}	5.967 ± 0.058^{a}	5.900 ± 0.100^{a}
4	6.259 ± 0.088^{b}	5.995 ± 0.079^{a}	5.898 ± 0.079^{a}
5	6.272 ± 0.109^{a}	7.099 ± 0.135^{b}	6.403 ± 0.088^{a}

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

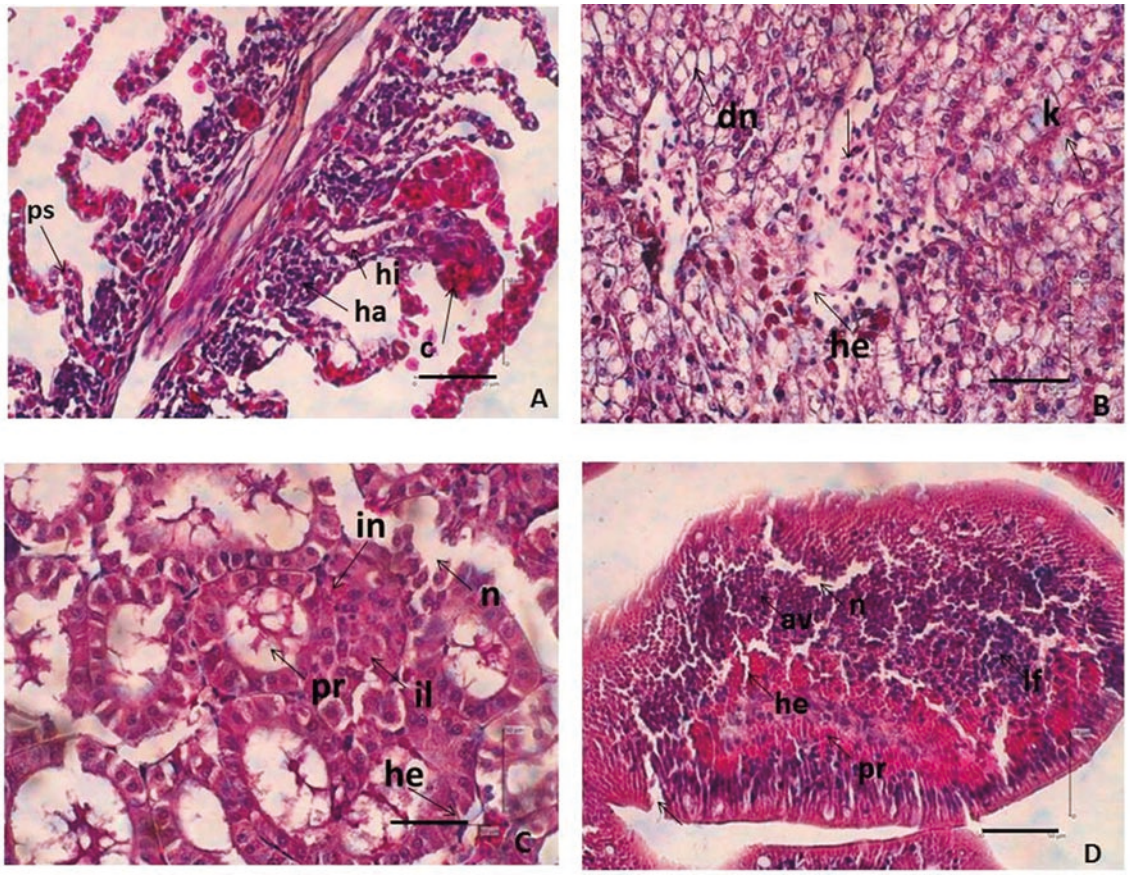

Figure 2. Organ histopathology of 3-month-old male tilapia fish (n = 3) (H & E staining, scale bar = 50 μm). (A) gill, (B) liver, (C) kidney, and (D) intestine. Note: ha = hyperplasia; hi = hypertrophy; c = clubbing; ps = bending cell; he = haemorrhage; k = congestion; dn = degeneration of nucleus; il = infiltrating lymphocytes; in = neutrophil infiltration; n = necrosis; pr = inflammation; av = intestinal villi atrophy; lf = lymphoid follicles; pr = inflammation.

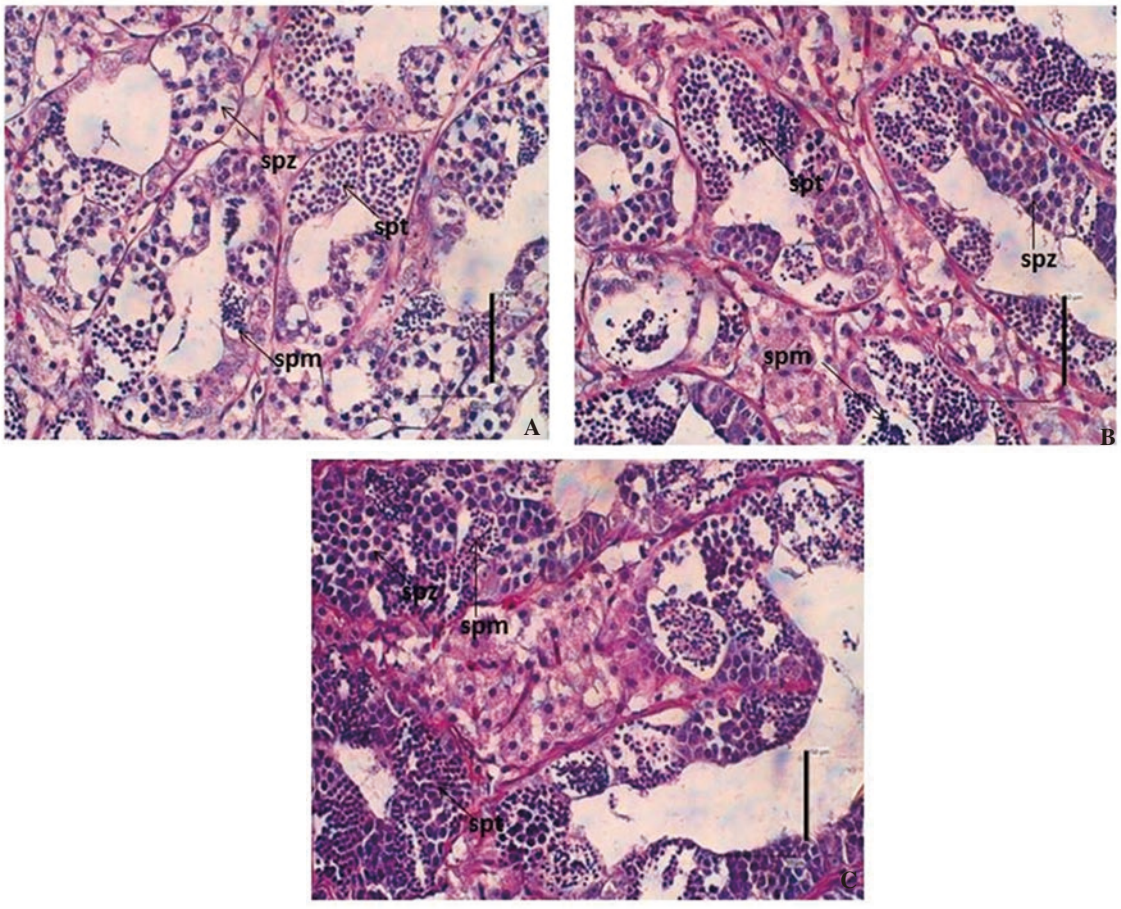

Figure 3. Testicular histology of 3-month-old male tilapia (n = 3); normal fish (A), orally methyltestosterone-treated fish (B), and methyltestosterone-immersed fish (C), spm=spermatocytes; spt=spermatid; spz=spermatozoa. (H & E, bar scale = 50 μm).

4. Discussion

Hormonal activities are affected by three stereochemical aspects, *i.e.*, location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration α or β, trans and/or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It is caused by the presence of bacteria in the gastrointestinal tract that oxidizes cluster 17β-hydroxy to be inactive17β-keto. Therefore, it is necessary to add an alkyl group on 17th carbon to become C17 α . This prevented the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17 α -MT compound has more activity in the body, but it could cause residue. The 17 α -MT activity has half the strength of testosterone activity due to the length of C-chain alkyl groups, and then it would decrease androgenic activity. Otherwise, it would increase its toxicity.

The 17 α -MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts[20,21]. Exposure to synthetic chemicals and their residue is a risk for human and wildlife health[3,22]. Based on serum MT concentration of males on the 3rd month, orally sex-reversed tilapia had more raising concentration than other treatments. However, in the 4th and 5th months, the MT concentration had decreased every month[3,22]. The orally MT-treated fish would contain MT only in the initial 5 months[23].

MT concentration was higher in the flesh compared to in the serum. High enough MT concentration was found in the muscle and flesh[24,25], because the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT to accumulate in the flesh every month. In the research of Pandian and Kirankumar[25], exogenous steroid remnants of 5 μg/kg in fish were a too risk to humans. Endogenous testosterone hormone produced on the testes was 5.2 μg/kg[26], whereas tilapia had endogenous testosterone and estradiol of 3 μg/kg, respectively[25]. Normal fish have higher MT residue value than MT-treated fish as shown in this study. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

Several limitations of this study are including: a) unable to measure specifically the MT residual concentration between introduced hormone (exogenous) and endogenously hormone by the fish, and b) unable to measure the MT residual concentration in younger fish age. Therefore, in the future, both of these limitations are our concern for further studies.

Gill layouts that were outside and directly related to water would be the first affected by the polluted water environment. The food already digested in the intestines would be circulated by blood to the liver and kidneys. The liver was the largest organ responsible for metabolism. Kidneys had functioned as a hyperosmotic regulator[27]. In 3-month-old fish, the toxicity to organs is still visible.

The early stage of damage caused by gill irritation has accompanied the increasing of the mucous cells at the bottom of epithelia with causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached. Gill lamella looked larger than normal which was caused by cell enlargement (hypertrophy), and it looked unclear between the primary and secondary lamellas. According to previous studies[17,28,29], hyperplasia may occur due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination has characterized by a very dense accumulation of red blood cells in the blood vessels, which would block blood vessels (congestion), while edema of lamella looks like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near to the lamella bottom (basal hyperplasia), and then the whole room of interlamellar was filled by new cells which showed like a baseball bat[27,30].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be returned to normal. Necrosis could not be cured, so if it exposed the tissue activity continuously, then it would decrease cell activity, causing the cells to lose some parts even to death[31,32]. Congestion was preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoid to widen that accumulated blood and hemorrhage. According to the research of Robert[30], congestion occurred by the entry of toxic substances into the heart. Hemorrhage was the flow of red blood cells out of the central vein.

Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine[33,34], which causes a greater concentration of toxic substances in this area and causes damage to the central vein. A sinusoid is a small capillary that separated the fundamental of the structural unit with tubule or trabeculae (biliary hepatocytes surrounded by central parenchyma)[33,35]. The liver had an enzyme for drug metabolism which is one of the most damaged organs but is very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged. But, it was still capable of regenerating and could even be cured if the damage was lost or destroyed[34].

The infected kidneys were swelling, which was an indication of an inflammatory process that may cause necrosis[35]. Inflammation was an indication of increased lymphocytes and macrophage or neutrophil cell numbers. Kidneys were pollutant-responsive organ to indicate histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach[36]. Changes that often occurred in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression[35–37].

The intestine damage is signed by inflammation. The inflammation or swelling of cells has a reversible characteristic that exposed to the toxic substances in a short period, the cell would return to normal, but if exposed to the toxic substances for a long time, the cell was not able to tolerate damage caused by toxin substances[38]. Melano-macrophage was caused by inflammation which was followed by erosion of the intestinal villi, hemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would disturb the absorption of important substances so that that fish would suffer from malnutrition. In intestinal organs, there were cell swelling, microvillicell membrane fused, lysis, intestinal vacuum and intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances[21]. Acute intestinal conditions were caused by viruses, parasites, bacteria, algae, and intestinal mucosa. Toxic chemicals could be removed by using mucous epithelial cells that coiled together with the thickening chromatin and cytoplasmic eosinophils[30]. MT concentrations of serum and flesh have not exceeded the limit (5 μg/L or 5 μg/kg) due to the estimated residual synthetic steroid in the fish body of 5 μg/kg. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histopathological figure of the 4- and 5-month-old fish to determine whether there is a recovery in the fish organ after the cessation of synthetic hormone.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authors' contributions

Dewi Nurmalita Suseno contributes to literature search, clinical and experimental studies, data analysis, and manuscript preparation. Epy Muhammad Luqman contributes to arrange the definition of intellectual content, data analysis, and manuscript review. Mirni Lamid contributes to arrange definition of intellectual content and statistical analysis. Akhmad Taufiq Mukti contributes to conceptualization, research design, arrange definition of intellectual content, data acquisition and analysis, and manuscript editing and review. Muhammad Agus Suprayudi contributes to arrange the definition of intellectual content, manuscript review, and guarantor.

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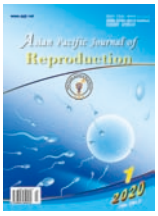

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Residual impact of 17α-methyltestosterone and histopathological changes in sex-reversed *Oreochromis niloticus*

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ABSTRACT

Objective: To examine sex reversal both by oral and by immersion using 17α-methyltestosterone on the methyltestosterone residual concentration and the organ histopathology of tilapia fish.

Methods: This study used oral and immersion treatment methods for sex reversal of tilapia fish and used normal fish as the control and each treatment was repeated 4 times. Dosages of 17α-methyltestosterone 60 mg/kg feed and 0.5 mL/L of 17α-methyltestosterone were used for oral and immersion methods, respectively. In the first step, tilapia fry were reared at 100 L aquaria, with a density of 1 fish/L for 2 months. In the next step, male tilapias were reared at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with a density of 30 fish/happa for 3 months. The methyltestosterone residual concentrations were analyzed by one-way analysis of variance and Duncan's multiple range tests, while organ histopathology was analyzed by descriptive method.

Results: Residual concentrations in the serum of methyltestosterone-treated fish were significant lower than that in normal fish, especially in 4- and 5-month-old tilapias with averages of less than 5 mg/L, while in normal fish was more than 5 mg/L. In the flesh, methyltestosterone residual concentrations showed relatively no significant differences between oral and immersion treatment groups and methyltestosterone-treated fish remained lower compared to normal fish, except in 5-month-old tilapia. Methyltestosterone-treated tilapia indicated histopathological changes on gill, liver, kidneys, and intestine organs.

Conclusions: Sex reversal either by oral or by immersion has methyltestosterone residual concentration, but does not exceed the limits (5 mg/L or 5 mg/kg) of synthetic steroid on the fish body, although methyltestosterone causes histopathological changes on gill, liver, kidneys, and intestine organs.

KEYWORDS: 17α-methyltestosterone; Residue; Organ histopathology; Tilapia; Sex reversal method

1. Introduction

Sex reversal both by oral and by immersion using synthetic steroids proved to be a simple, easy, and highly effective technology[1]. Androgenic anabolic steroid hormones such as 17α-methyltestosterone (17α-MT)[2,3] is a derivative of testosterone[4], which potentially increases sexual developmental in males[3]. The 17α-MT-immersed tilapia larvae produce males of 91.6%-98.3% [5,6], however, oral treatment of 60 mg/kg feed produces males of 93.7%[7], 97.7%[8], even reaches up to 100% males[9].

Synthetic steroid hormone would enter through the blood vessels in the body and then it was modulated by the brain and pituitary hormones[10]. Steroid hormone was synthesized in either the liver or the kidneys[11], and subsequently, it would produce androstenedione which consists of 17β-estradiol and testosterone. If testosterone has increased, then the gonads would be immediately addressed to the male sex, but 17α-MT has characteristic that it is difficult to be absorbed within the body and it will also contaminate environment[12].

The utility of hormones in aquaculture production was often debated by researchers due to the potential toxicity on human health (a carcinogenic and endocrine disorder) as well as the danger to environment[1,3,13-15]. The group of anabolic steroids

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(including 17α-MT) based on the decision of Ministry of Marine Affairs and Fisheries, Republic of Indonesia (number KEP.52/MEN/2014) has been banned because the hormones were harmful to fish, environment, and human. This study expected to prove the presumption that has been the subject of debate in the fish farmer community that the use of 17α-MT at any dose produces dangerous and toxic residues when consumed by humans and the released into the environment, as well as the debate among researchers and to address concerns that have existed in the community and policy makers that the use of 17α-MT in certain doses is still safe and does not contain dangerous residues of concern so far. So that the regulation can be revised again for the advancement of aquaculture while maintaining a sustainable environment and human health that consumes cultured fish. Therefore, the aims of the study were to examine sex reversal both by oral and by immersion using 17α-MT on the MT residual concentration and the organ histopathology changes of tilapia.

2. Materials and methods

2.1. Test animal

The test animal used was Nile tilapia (*Oreochromis niloticus*). Tilapia fry were produced by artificial fertilization and controlled incubation.

2.2. MT treatments

MT treatment by oral method was started 3 days after hatching with using 17α-MT (Argent) dose of 60 mg/kg feed. The oral treatment method lasted for 28 days. Immersion method using dose of 0.5 mg/L of 17α-MT was conducted to 10-day-old Tilapia fry and repeated in 13-day-old Tilapia fry for 3 h, respectively[8]. Treatment groups (namely MT-treated fish, both by oral and by immersion) and normal fish as control were repeated 3 times, respectively with a density of 100 fish/replicate/treatment, so the total of fish, both treated and normal were 900 fish.

2.3. Fish rearing

In the initial step, fish were reared at 100 L aquaria, with a density of 1 fish/L for 2 months, separately in each treatment group. Fish was fed on commercial pellet content of 40% crude protein, 3 times daily, at satiation. Sex was determined on 2-month-old fish through manual observation of genitalia for all fish, and gonad preparation. To verify the sex from genitalia observation, gonad was obtained from 10 fish/replicate/treatment by using the squash method with acetocarmine dye according to Mukti[8]. Based on 17α-MT hormone treatment either by oral or by immersion and verify the sex by fish genetalia observation and followed by gonad preparation shows male of 97%-98% and female of 2%-3%[8]. Then, male fish of 3 treatments were selected for further study.

In the next step, a total of 360 male tilapias used in this study for 3 treatments (120 fish/treatment) were reared separately at happa (net cage) of (2×1×1) m^3 size in the controlled pond, with the density of 30 fish per happa or replicate, respectively for 3 months. Each treatment was repeated 4 times. Fish was fed on commercial pellet content of 32% crude protein, 3 times daily, at-satiation.

2.4. Sampling

Fish sampling was done in the 3rd, 4th, and 5th months as much 3 fish/replicate/treatment, respectively for residue test. 3-month-old fish were used for histology preparation. Fish were anesthetized by using MS222 of 1 mg/L according to Gogal *et al*[16]. Serum (1 mL) was collected according to Atli *et al*[17], and flesh (10 g) of fish was collected to do testing of residues.

2.5. Measurement of MT residue

MT residue, both the serum and the flesh were measured by the sandwich enzyme-linked immunosorbent assay method using fish MT kit (cat number E0103Fi; Bioassay Technology Laboratory, Shanghai, China). Previously, the sample and the reagents were stored at a temperature of 18-25 ℃[3].

2.6. Histology preparation

Fish was carefully dissected on abdominal part according to Wu *et al*[18] and gill, liver, kidneys, intestine, and gonad organs were collected and stored in the 50 mL tubes which consisted of buffer neutral formalin, with the ratio of 1:2 at room temperature. Histology processes were conducted according to the standard operational procedure, generally with slight modification[19]. The flow chart of the study was shown in Figure 1.

2.7. Statistical analysis

Data of MT residual concentrations were analyzed statistically by using analysis of variance (ANOVA) with SPSS ver.10 software. Significant ANOVA was followed by Duncan's multiple range test, while organ histopathology was analyzed descriptively. Data were expressed as mean ± standard deviation (mean ± SD). *P*–value < 0.05 was considered as statistical difference.

2.8. Ethical approval

The study was approved by the Animal Care and Use Committee of Brawijaya University; the protocol number was 985/8.8.2017.

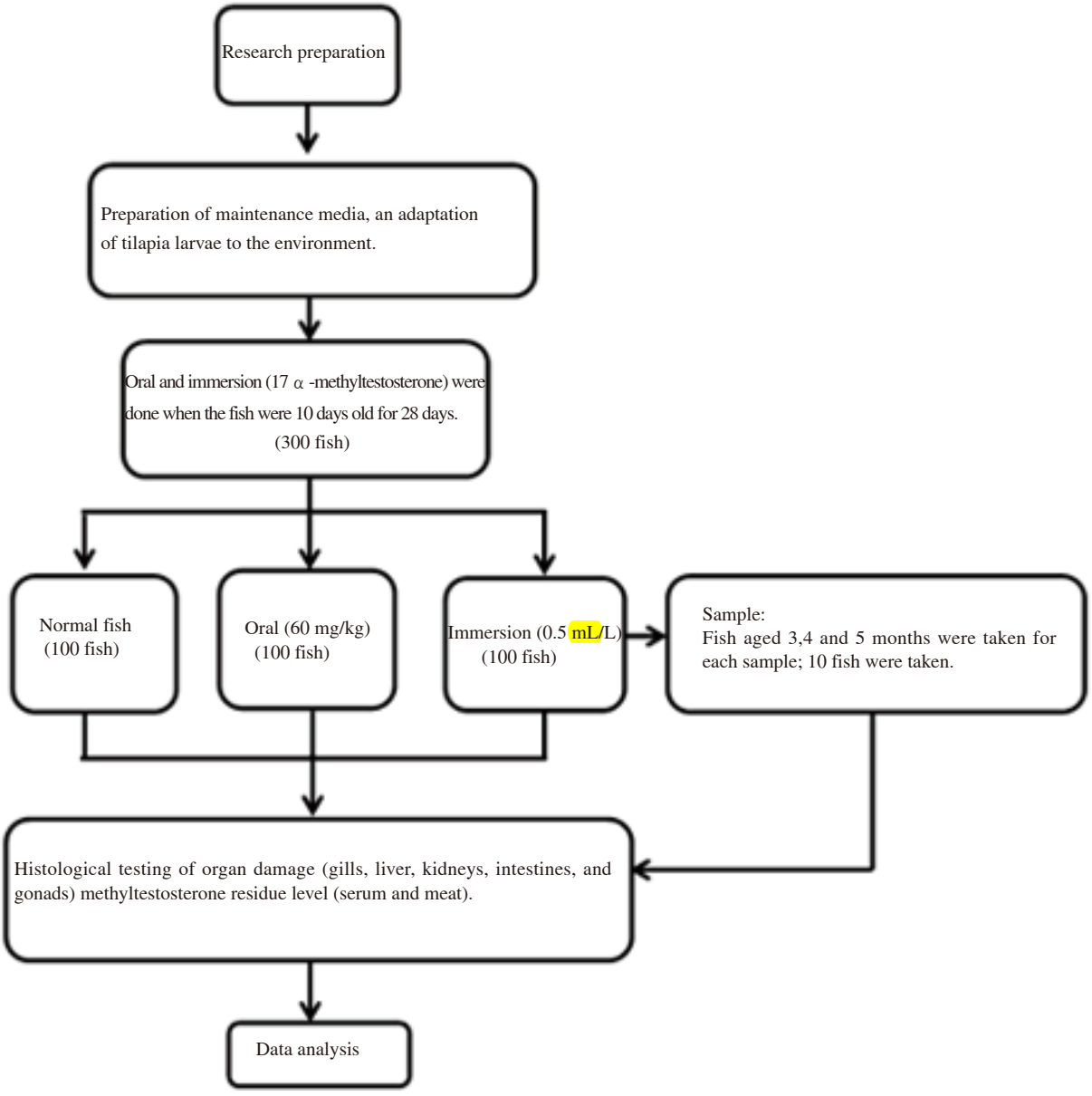

Figure 1. Flow chart of the study.

3. Results

3.1. MT residual concentrations

MT residual concentration in the serum of MT-treated male tilapia, both by oral and by immersion, was decreased on 4th month while increased again in 5th month, the normal male fish was increased from 4th month while slightly decreased in 5th month (Table 1). On 4th and 5th months, the MT residue concentrations were lower in both oral and immersion groups comparing with that of the normal group (P both <0.05).

In the flesh, MT residual concentration showed relatively no significant difference between the oral and immersion treatment groups on 4th month, but the MT residual was significant higher in oral treatment group than that in the immersion group. MT-treated male tilapia remained lower than normal male tilapia in the 3rd and 4th months, except in the 5th month. However, the result showed that all males had increased MT residue in the 5th month comparing with that of the 4th months (Table 2).

3.2. Organ histopathology

MT-treated male tilapias showed histopathology changes in gill, liver, kidneys, and intestine organs (Figure 2). In the gill, such as hyperplasia was found in the bottom secondary lamella. Hypertrophy appeared on the lamella stem due to the occurrence of containment. Clubbing occurred at the end of the primary lamella, which was caused by the existence of retention, so edema appeared on the lamella (Figure 2A). The liver showed congestion, hemorrhage, and cell atrophy (Figure 2B). Congestion was redder due to contained erythrocytes. Atrophy was shown by the reduction cell size of Kupper, which made sinusoid widen and made vacuoles degenerate. Congestion caused sinusoidal erythrocytes to wide. Degeneration of liver cells made vacuoles

enlarge. Normally, the liver organ did not have damage. Kidneys seem hemorrhage, infiltration of lymphocytes, and neutrophils, inflammation, and necrosis (Figure 2C). The infiltration presence of lymphocytes and neutrophils caused inflammation. The intestine has look atrophy, intestinal villi hemorrhage, lymphoid follicles, and melanomacrophage (Figure 2D). The occurrence of hemorrhage led to the atrophy and melanomacrophage, so finally, it caused erosion and hemorrhage and necrosis of the intestinal villi. On the other hand, testicular histology (Figure 3) used to observe spermatogenesis or testicular development and may be histopathology change in different treatment of 3-month-old fish. This study showed no difference in testicular between normal fish (Figur 3A) and MT-treated fish, both oral (Figure 3B) and immersion (Figure 3C).

Table 1. Methyltestosterone residual concentrations (mg/mL) of serum in different age of male tilapia.

Ages of tilapia (month)	Treatments		
	Normal	Oral	Immersion
3	4.403 ± 0.058^{8a}	5.243 ± 0.080^{b}	4.431 ± 0.029^{a}
4	5.117 ± 0.057^{c}	4.171 ± 0.051^{b}	3.874 ± 0.038^{a}
5	5.105 ± 0.079^{c}	4.266 ± 0.050^{a}	4.450 ± 0.054^{b}

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

Table 2. Methyltestosterone residual concentrations (mg/g) of the flesh in different age of male tilapia.

Ages of tilapia (month)	Treatments		
	Normal	Oral	Immersion
3	6.061 ± 0.094^{a}	5.967 ± 0.058^{a}	5.900 ± 0.100^{a}
4	6.259 ± 0.088^{b}	5.995 ± 0.079^{a}	5.898 ± 0.079^{a}
5	6.272 ± 0.109^{a}	7.099 ± 0.135^{b}	6.403 ± 0.088^{a}

Data are expressed as mean ± SD. Different superscripts (a, b,c) in the same row show significant difference (P<0.05).

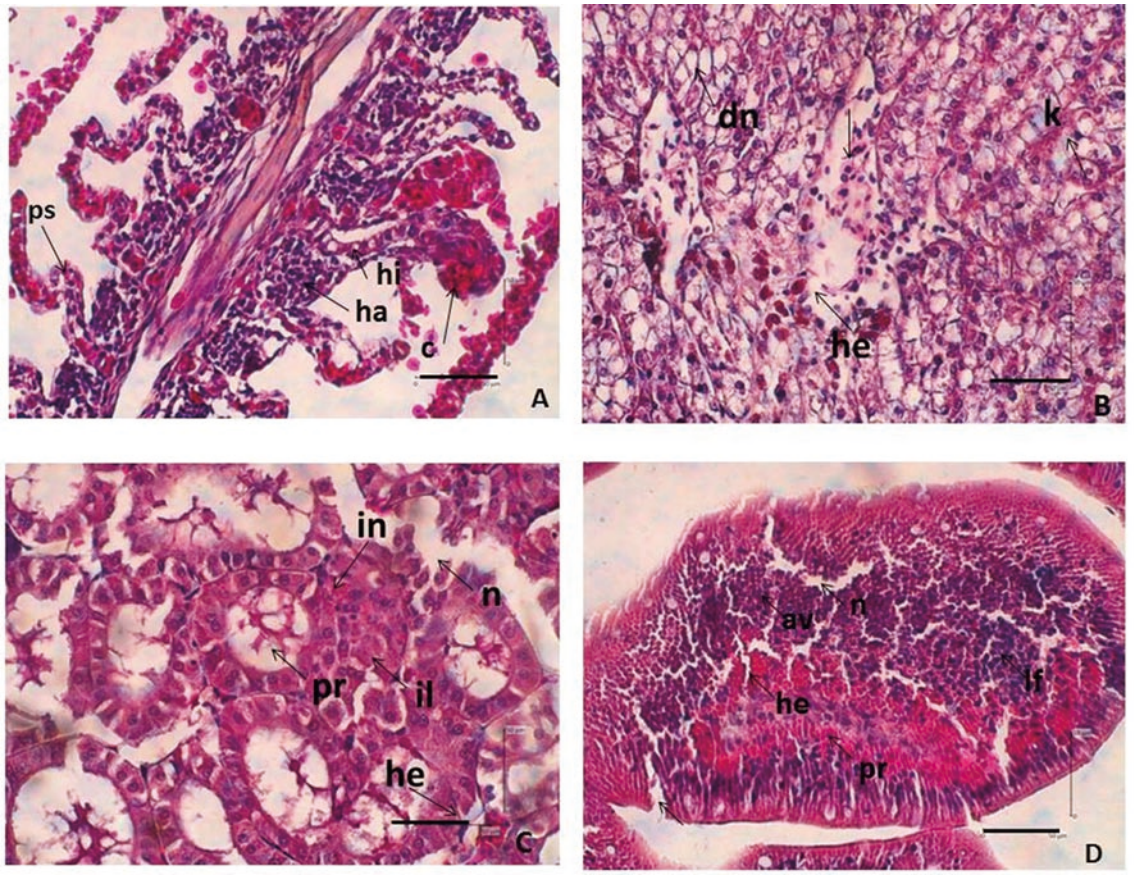

Figure 2. Organ histopathology of 3-month-old male tilapia fish (n = 3) (H&E staining, scale bar = 50 µm). (A) gill, (B) liver, (C) kidney, and (D) intestine. Note: ha = hyperplasia; hi = hypertrophy; c = clubbing; ps = bending cell; he = haemorrhage; k = congestion; dn = degeneration of nucleus; il = infiltrating lymphocytes; in = neutrophil infiltration; n = necrosis; pr = inflammation; av = intestinal villi atrophy; lf = lymphoid follicles; pr = inflammation.

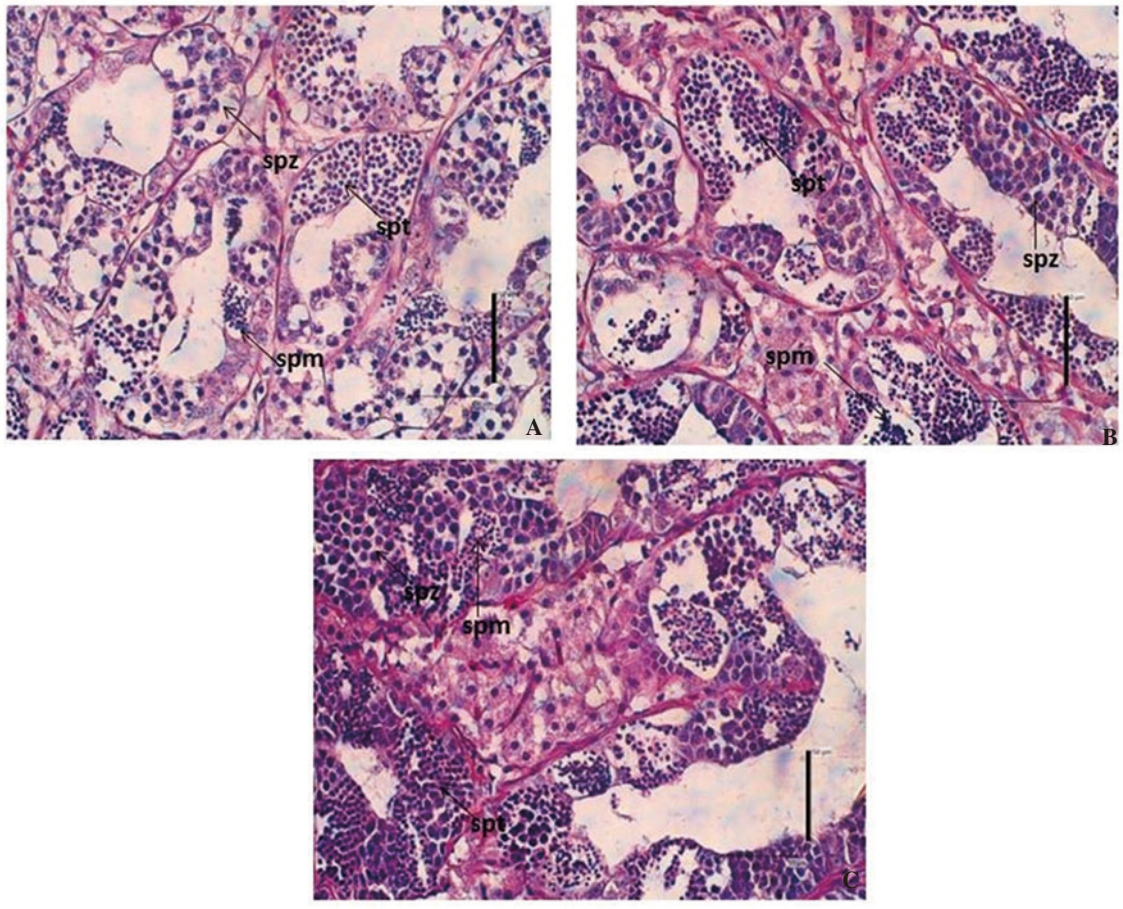

Figure 3. Testicular histology of 3-month-old male tilapia (n = 3); normal fish (A), orally methyltestosterone-treated fish (B), and methyltestosterone-immersed fish (C), spm=spermatocytes; spt=spermatid; spz=spermatozoa. (H&E, bar scale = 50 μm).

4. Discussion

Hormonal activities are affected by three stereochemical aspects, *i.e.*, location of the cluster on the ring, axial and or equatorial positions, cluster, the configuration α or β, trans and/or isomer, and cyclohexane ring conformations. Testosterone is a hormone that has a short activity time due to fast absorbance in the digestive tract and rapidly undergoes hepatic degradation. It is caused by the presence of bacteria in the gastrointestinal tract that oxidizes cluster 17β-hydroxy to be inactive17β-keto. Therefore, it is necessary to add alkyl group on 17th carbon to become C17 α . This prevented the conversion of 17β-hydroxy metabolism to be 17β-keto, so the 17 α -MT compound has more activity in the body, but it could cause residue. The 17 α -MT activity has half the strength of testosterone activity due to the length of C-chain alkyl groups, and then it would decrease androgenic activity. Otherwise, it would increase its toxicity.

The 17 α -MT compounds could be transferred to live feed or water. Chemical substances had naturally incorporated into living organisms in several ways, through both the digestive and respiratory tracts[20,21]. Exposure to synthetic chemicals and their residue is risk for human and wildlife health[3,22]. Based on serum MT concentration of males on the 3rd month, orally sex-reversed tilapia had more raising concentration than other treatments. However, in the 4th and 5th months, the MT concentration had decreased every month[3,22]. The orally MT-treated fish would contain MT only in the initial 5 months[23].

MT concentration was higher in the flesh compared to in the serum. High enough MT concentration was found in the muscle and flesh[24,25], because the MT metabolite has been absorbed into the muscle and flesh of fish, thus causing the MT to accumulate in the flesh every month. In the research of Pandian and Kirankumar[25], exogenous steroid remnants of 5 000 ng/kg in fish were too low risk to humans. Endogenous testosterone hormone produced on the testes was 5 200 ng/kg[26], whereas tilapia had endogenous testosterone and estradiol of 3 000 ng/kg, respectively[25]. Normal fish have higher MT residue value than MT-treated fish as shown in this study. We suspect this is related to the reproductive cycle or period of Nile tilapia. Normally, Nile tilapia at the 4-month-old has entered the period of reproduction and spawning, so that seen an increase in hormone levels in blood serum. As is known during entering reproduction or spawning, hormone levels in the body increase and will drop back after spawning, while monosex-treated fish, although it looks the same as normal, the body's energy is preferred in increasing somatic growth compared to reproduction, so we suspect that this is one of the factors that causing male monosex-treated Nile tilapia has a larger body size than normal male tilapia.

Several limitations of this study are including: a) unable to measure specificaly the MT residual concentration between introduced hormone (exogenous) and endogenously hormone by the fish, and b) unable to measure the MT residual concentration in younger fish age. Therefore, in the future, both of these limitations are our concern for further studies.

Gill layouts that were outside and directly related to water would be the first affected by the polluted water environment. The food already digested in the intestines would be circulated by blood to

the liver and kidneys. The liver was the largest organ responsible for metabolism. Kidneys had functioned as a hyperosmotic regulator[27]. In 3-month-old fish, the toxicity to organs is still visible.

The early stage of damage caused by gill irritation has accompanied the increasing of the mucous cells at the bottom of epithelia with causing a thickening of the secondary lamella epithelium so that the secondary lamella enlarges due to the secondary lamella attached. Gill lamella looked larger than normal which was caused by cell enlargement (hypertrophy), and it looked unclear between the primary and secondary lamellas. According to previous studies[17,28,29], hyperplasia may occur due to chemical stimuli from pollutants, environmental pollution, parasites, and bacterial infections. Contamination has characterized by a very dense accumulation of red blood cells in the blood vessels, which would block blood vessels (congestion), while edema of lamella looks like an empty white space that causes blocking. Clubbing occurred because of the thickening of epithelial tissue located near to the lamella bottom (basal hyperplasia), and then the whole room of interlamellar was filled by new cells which showed like a baseball bat[27,30].

Degeneration was the early stage of vacuole damage in the liver. Vacuole degeneration was reversible, so when exposed to toxic substances and end administration of MT, cells could be returned to normal. Necrosis could not be cured, so if it exposed the tissue activity continuously, then it would decrease cell activity, causing the cells to lose some parts even to death[31,32]. Congestion was preceded by degeneration of liver cells in which an enlarged vacuole was filled with erythrocytes that cause sinusoid widen that accumulated blood and hemorrhage. According to the research of Robert[30], congestion occurred by the entry of toxic substances into the heart. Hemorrhage was the flow of red blood cells out of the central vein.

Sinusoidal and central venous damage occurred due to numerous blockages of blood vessels in the stomach and central intestine[33,34], which causes a greater concentration of toxic substances in this area and causes damage to the central vein. A sinusoid is a small capillary that separated the fundamental of the structural unit with tubule or trabeculae (biliary hepatocytes surrounded by central parenchyma)[33,35]. The liver had enzyme for drug metabolism which is one of the most damaged organs but is very resistant to viral or bacterial infections and foreign substances that enter through the absorption in the intestine. It was known that nearly 80% of the liver cells were damaged. But, it was still capable of regenerating and could even be cured if the damage was lost or destroyed[34].

The infected kidneys were swelling, which was an indication of an inflammatory process that may cause necrosis[35]. Inflammation was an indication of increased lymphocytes and macrophage or neutrophil cell numbers. Kidneys were pollutant-responsive organ to indicate histopathological damage. Therefore, the kidneys were the targeted organ for the biomonitoring approach[36]. Changes that often occurred in the kidney are inflammation, necrosis, thickening of the core, hyperplasia, hypertrophy epithelial cells, hydropic vacuolation, and renal tubular regression[35–37].

The intestine damage is signed by inflammation. The inflammation or swelling of cells has a reversible characteristic that exposed to the toxic substances in a short period, the cell would return to normal, but if exposed to the toxic substances for a long time, the cell was not able to tolerate damage caused by toxin substances[38]. Melano-macrophage was caused by inflammation which was followed by erosion of the intestinal villi, hemorrhage, and atrophy leading to necrosis. Erosion and villus of the intestine with considerable damage would disturb the absorption of important substances so that that fish would suffer from malnutrition. In intestinal organs, there were cell swelling, microvillicell membrane fused, lysis, intestinal vacuum and intestinal villi erosion which suffered severe injuries to rupture caused by toxic substances[21]. Acute intestinal conditions were caused by viruses, parasites, bacteria, algae, and intestinal mucosa. Toxic chemicals could be removed by using mucous epithelial cells that coiled together with the thickening chromatin and cytoplasmic eosinophils[30]. MT concentrations of serum and flesh have not exceeded the limit (5 000 ng/L or 5 000 ng/kg) due to the estimated residual synthetic steroid in the fish body of 5 000 ng/kg. Influences on histopathology of gill, liver, kidneys, and intestine organs are found with varying degrees of damage because there are remaining synthetic hormones left in the body that cause organ damage. Further work is another safer natural material to replace the performance of the alkyl group as well as the histopathological figure of the 4- and 5-month-old fish to determine whether there is a recovery in the fish organ after the cessation of synthetic hormone.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authors' contributions

Dewi Nurmalita Suseno contributes to literature search, clinical and experimental studies, data analysis, and manuscript preparattion. Epy Muhammad Luqman contributes to arrange definition of intellectual content, data analysis, and manuscript review. Mirni Lamid contributes to arrange definition of intellectual content and statistical analysis. Akhmad Taufiq Mukti contributes to conceptualization, research design, arrange definition of intellectual content, data acquisition and analysis, and manuscript editing and review. Muhammad Agus Suprayudi contributes to arrange definition of intellectual content, manuscript review, and guarantor.

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At 2020-01-02 00:03:19, "taufiq mukti" <atm_mlg@yahoo.com> wrote:

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1	As we plan to publish your paper next week, please carefully proofread the full text again before publication. Authors' names and affiliations should be checked, and make sure they are correct. Once the article is published online, the content in it cannot be changed any more. All the content should be correct. If there are no mistakes, please confirm this version as the final version which will be published online next week.	We have proofread and checked the full text of the article. There are no mistakes. We also confirmed that the article as the final version to publish. Thank you very much.	

Fwd: [apjr]:Acknowledgment for re-revised manuscript:apjr_52_18

Dari: dewi nurmalita Moer (dnesmoer@gmail.com)

Kepada: atm_mlg@yahoo.com

Tanggal: Senin, 20 Januari 2020 pukul 03.33 GMT+7

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Dari: **Asian Pacific Journal of Reproduction** <editor.apjr@journalonweb.com>
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To: <dnesmoer@gmail.com>

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Manuscript no.: apjr_52_18
Dear Miss. Suseno
Asian Pacific Journal of Reproduction has received your revised manuscript entitled '"Residual impact of 17a-methyltestosterone and histopathological changes in sex-reversed Nile tilapia (Oreochromis niloticus)"..' The manuscript will be re-evaluated by concerned referees before final decision on its suitability for publication. We will get back to you within four weeks.
We thank you for submitting your valuable research work to Asian Pacific Journal of Reproduction.
With warm personal regards,
The Editorial Team
Asian Pacific Journal of Reproduction

Message sent on Sunday, January 19, 2020
Please add **editor.apjr@journalonweb.com** as a contact in your E-mail c lient to ensure that this mail is not considered as a junk mail.

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