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Research Article

Accelerated of Sex Reversal use 17α-methyltestosterone Induced Female, Orange-Spotted Grouper *Epinephelus coioides*

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Abstract

The occurance of hermaphrodites in grouper fish causes a scarcity of male parents, so an alternative is needed to accelerate sexchange to male at a young age. The present study was expected to scrutinize the mechanisms of sex-change in fish in the early change process, and whether the testis converted from immature ovary using 17α-methyltestosterone (MT) would recover after the termination of MT treatment. MT-induced sex-change and 5-aza-2'-deoxycytidine (5-Aza) were connected as DNA methylation inhibitors to comprehend the alternation of gonadal soma cells. The orange-spotted groupers were used at the developmental ages and fed a diet containing MT at 50 mg/kg for three months and then a normal diet for a month. In the first week and second week fish injected with 5-Aza intraperitoneally during the MT-oral administration. Most of the fishes in the control group had immature ovaries, but all the females fed with MT, had immature spermatogenesis. However, one month after the withdrawal of MT treatment, the sex of the fish returned to female-like even though the fish have undergone MT-induced masculinization. This outcome demonstrates precocious sex-change from under yearling, orange-spotted grouper utilizing oral MT treatment is impermanent. All the females of 5-aza treatments showed no spermatogenic cells. In this study, lower growth rates were demonstrated by the MT-treated groups. The impact of this metabolic change was clear after the end of the hormone oral administration since the decreased growth of the groups treated for three months.

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1. Introduction

In fish culture, control of proliferation is vital to deal with the number and timing for business purposes, i.e. for the seed stock production, selective breeding, growth rate, feed efficiency, meat quality, and biosecurity (Overturf, 2009). Thus, need to be controlled by the farmers and even between the fragments in the same business. No matter the encouragement for the aquaculturists wants to alter the reproductive events, progress in gaining control over reproductive processes, is based on our gaining comprehension of mechanisms that control these processes.

A molecular method has been turned into an instrumental in numerous latest advances in the point of the regenerative control system in fish (You *et al*., 2020; Sundaray *et al*., 2022; Ajani *et al*., 2022). The studies of reproductive or sex control and natural sex-reversal mechanisms in fishes are not just of academic background to the understanding of sex determination and sex differentiation, but additionally of economic value in aquaculture and fish farming (Chan and Yeung, 1983). There is considerable diversity in reproductive physiology among fish species and an understanding of the basic reproductive processes with varying modes of reproduction is required to be aware of these primary commonalities and differences (Overturf, 2009). The interesting phenomenon among teleost fishes is the existence of several different reproductive modes. The process itself has several independent origins, even though in each case it is initiated and (or) regulated by gonadal steroids (Frisch, 2004).

The orange spotted-grouper, *Epinephelus coioides* are commercially important coral reef fishes because of their excellent flavor and high price (Nakamura *et al*., 2007). The price of this fish in the central part of Indonesia to the east is around IDR 65,000 (Achmad *et al*., 2022). They are widely distributed throughout the tropical and subtropical waters of the world and are commercially important and highly regarded as a favorite marine food fish (Yeh *et al*., 2003). It belongs to the protogynous hermaphrodite fish species and is widely cultured in Asia including southern mainland China, Hong Kong, and Taiwan, and the mariculture of the orange-spotted grouper is at an experimental scale in Hong Kong (Liu and Sadovy, 2008). In orange-spotted grouper, *Epinephelus coioides* the male characteristics were reduced, and male-to-female sex-change occurred after aromatase inhibitor (AI)/MT-termination in the AI-and MT-induced maleness (3 months of oral administration) (Wu *et al*., 2015). This phenomenon also occurred in the protandrous black porgy, *Acanthopagrus schlegeli*, which when treated with estradiol, at high doses, induce regression of testicular tissue and stimulate the development of ovarian tissue (Chang *et al*., 1995). Based on this, the gonadal differentiation can be reversed, particularly in hermaphroditic species, but also in gonochorists (Devlin and Nagahama, 2002).

The hypophyseal and pituitary levels control give influence in mediating environmental cues for sex change in orange-spotted grouper and MT treatment could induce sex-change, but it is not stable because fish reverted to female after MT termination, and maintenance of secondary sex determination is correlated with sex steroid. The 5-aza could not promote ovarian differentiation in the sex-changing fish via other mediators in the gonadal differentiation signaling pathways, due to lower doses. However, studies about this is still limited.

The present study was expected to know the instrument of sex-change in groupers in the early change process, and whether the testis that is turned around from immature ovary using MT would recover after the termination of MT treatment. We connected MT-induced sex-change and 5-aza-2-deoxycytidine as DNA methylation inhibitors to comprehend the alternation of gonadal soma cells. We also want to know the proliferating activity and alteration of function in the female-to-male sex change.

2. Materials and Methods

2.1 Experimental Fish

The orange-spotted groupers in development ages (4 months old; n=200; body weight 10.48 ± 1.58 g; body length 9 ± 0.57 cm) were used in the experiments obtained from local farmers in the southern part of Taiwan, Republic of China. All fish used in this study were cultured in seawater tanks at the Department of Aquaculture, National Taiwan Ocean University, and treated in compliance with a protocol approved by the National Taiwan Ocean University animal care and Use Committee.

All experimental fish were acclimated in a pond at the university culture station in seawater with a natural light system (water temperature ranged from 24 to 28 °C). A recirculating system was designed for each 2.5-ton fiber-reinforced plastic (FRP) tank with a sand filter, and biofilter to decrease organic substances and feces. The fish were randomly divided into four groups (n=50) and each group was allowed to acclimate for approximately two weeks. The fish were fed ad libitum with a commercial food (Fwu Sou Feed Co., Taichung, Taiwan) twice a day.

2.1.1 Ethical approval

All fish used in this study were cultured and treated in compliance with a protocol approved by the National Taiwan Ocean University animal care and Use Committee. All animal experimental and rearing procedures were also handled in accordance to animal welfare under the national accreditation no. SNI 01-6488.1-2000 of Republic of Indonesia.

2.2 Method

The study was conducted at the Aquatic Laboratory Centre of Aquaculture Department and samples were carried out using the facilities at the Laboratory of Molecular Biology, Reproductive Physiology, and Endocrinology, Physiology in Aquatic Animals, Aquaculture Department, National Taiwan Ocean University, Keelung, Taiwan, Republic of China.

2.2.1 Experiment 1. Fish treated with MT-oral administration and induced with 5-aza-2'-deoxycytidine (DNA methylation inhibitor)

After initial acclimation, four batches of orange-spotted groupers were used for the experiment (n=50 fish per group) respectively as controls (without treatments), androgen hormone (MT, 17α-methyltestosterone, Sigma Co. Ltd) feed groups, 5-aza-2′-deoxycytidine (5-Aza, Sigma Co. Ltd) induce groups and MT feed+5-aza induce groups. To investigate the possible involvement of DNA methylation in gonadal differentiation of the orange-spotted grouper, we used DNA methylation inhibitor 5-aza, and the experimental fish were intraperitoneally injected with 1.0 mg/kg BW of 5-aza on days 7, 10, and day 14. For the MT feed preparation, 50 mg/kg of MT were dissolved in 95% ethanol, and then thoroughly sprayed into the diet (nearly 4 mL ethanol per 10 g feed). Ethanol was removed through evaporation at 40°C for about four hours (Navarro-Martin *et al*., 2011; Ribas *et al*., 2017). The treated feed was stored at 4°C until used for the feeding trial. Fish were fed ad libitum two times daily.

The gonad of the fish (n=10 fish per group each time sampling) was collected on day 0, day 30 (one month treatment), day 60 two months treatment), day 90 (three months treatment) and fixed in a 4% para-formaldehyde solution for the histological analysis. Before sampling, the fish were anesthetized in ethylene glycol monophenyl ether (0.05%) and put down by decapitation. Bodyweight (BW) and total length (TL) were measured.

A piece of gonad from every different group was collected for histological observation. Gonad tissues were fixed in a 4 % paraformaldehyde solution for more than 16 hours, subsequently transferred into flush water for 2 hours and 70 %, 80 %, and 90 % ethyl alcohol for 30 minutes each, and then stored in 100 % ethyl alcohol until further use.Moreover, fixed gonads were dehydrated and embedded in paraffin. They were embedded in paraffin wax (Sherwood Medical, St Louis, MO, USA) and sectioned at least 10 sections 5-6 µm thick using a microtome of each fish then mounted on slides (Humason, 1979; Blazer, 2002; Alonso-Fernandez *et al*., 2011).

Paraffin-embedded sections were treated with traditional haematoxylin and eosin (HandE) (Gill-2 Haematoxylin, Thermo Shandon, Pittsburgh, PA, USA; Eosin-Y Thermo Shandon) or trichrome staining according to the Gomori rapid 1-step trichrome method (Bancroft and Cook, 1994). The Stained sections were sealed with microscopy entellan (Merck, Darmstadt, Germany) and photographed with an optical microscope to determine their phenotypic sex. Each fish was categorized as male, female, or intersex. Fish were classified as interphase when both female and male germ cells were seen in the same gonad slice (Humason, 1979; Blazer, 2002; Alonso-Fernandez *et al*., 2011).

2.2.2 Experiment 2. Fish treated with MT and AI-oral administration and induced with 5-bromo-2-deoxyuridine (BrdU)

In order to consider the cell-sexual re-programming of the cells in the MT-terminated male-to-female sex-changed fish, the AI/MT feed in the MT fish was ended after three months of AI/MT-oral administration. Before the examination begins, the gonads of differentiated juvenile female fish were gathered to check the sex stage. Juvenile female fish reared up to seven months old were fed a diet containing AI/MT for three months, fish (n=8 fish, each in the treated and control groups). These treatments aim to impel the fish to experience sex change from female to male (Wu *et al*., 2015).

The feed contained AI (1,4,6-androstatriene-3,17-dione, ATD) fed to the fish at 20 mg/kg fish body weight (inhibited aromatase activity) and MT diet at 50 mg/kg body weight of fish (increased androgen levels). Furthermore, there are three groups were designated for the experiment. Group 1: The AI/MT terminated fish (day 0 is the day, to begin with, AI/MT termination) were injected with BrdU (0.3 mg/g of body weight) on day 6 and day 8, and gonadal tissues were collected on day 14 (two weeks). Group 2: The AI/MT terminated fish were injected with BrdU (0.3 mg/g of body weight) on days 16 and 18, and then the gonadal tissues were collected on day 28 (four weeks). Group 3: The AI/MT terminated fish were injected with BrdU (0.3 mg/g of body weight) on day 30 and day 32, and the

gonadal tissues were collected on day 56 (four weeks) (Wu *et al*., 2015). All the tissue samples were used to see the gene expression of the fish gonad at different developmental days by using qPCR analysis.

2.2.3 Microscopy

For HandE micrographs, bright and dark field images were obtained using an Olympus BX53 photo-microscope equipped with a DP72 digital camera, and micrographs were acquired in a TIFF or JPEG format with the Olympus Analysis software. The digital images were then edited with Adobe Photoshop software (San Jose, CA), but only the brightness and contrast results were adjusted**.**

2.2.4 Quantitative real-time PCR

In this study, we used the first-strand cDNA from the fish gonad and the specific primer for q-PCR analysis which consist of dmc1, nanos2, and sycp3 genes (Table 1). The characteristics of dmc1 and sycp3 are reported to be specifically expressed during the meiotic prophase in mammals (Nakamura *et al*., 2010). In mice, nanos2 expression in developing male germ cells is involved in preventing germ cells from entering meiosis (Tsuda *et al*., 2003). In medaka and zebrafish, nanos2 has been detected in adult undifferentiated germinal cells (Aoki *et al*., 2009) and more precisely, nanos2 is expressed in premeiotic oogonia that are proposed as the ovarian germ stem cells (Nakamura *et al*., 2010).

Table 1. The specific primer used in this study

Gene name		Sequence (5' - 3')
dmc1	F	AGACATCGCTGACAGGTTTAAT
	R	CTACAAAGTCCAACAGCTCCA
nanos2	F	TTCTGCAGGCACAACGG
	R	AGGTGTAGTTGTAGAGGATGGG
sycp3	F	GTTCAGACAGCAGCAGAAGA
	R	TCCTCCATGTTCTTCACAAACT
gapdh	F	CGACCCTCACTCCTCCCATCTT
	R	GCTGTAGCCGAACTCCGTTGTTC

Gene expression of the fish gonad at different developmental days (from 0 days; 2 weeks, 4 weeks, and 8 weeks after AI/MT termination) was quantified by q-PCR analysis. Q-PCR primers were designed for the respective genes using Primer Expression software (Applied Biosystem). Gene quantification of standards, samples, and controls was simultaneously conducted in a qPCR machine (CFX Connect Real-Time System; Bio-Rad) with IQTM SYBR Green Master Mix (Applied Biosystems, Foster City, CA) as a dsDNA minor-groove binding dye, forward, reverse primer, and water into each well of a MicroAmp® 96-well reaction plate. The respective standard curve of the log transcript concentration vs CT (the calculated fractional cycle number at which the PCR-fluorescence product is detectable above a threshold) was obtained (Han *et al*., 2018; Qin *et al*., 2019; Huang *et al*., 2021, 2023; Zhu *et al*., 2022).

Serial dilutions of the standard were prepared to detect the values from different amounts of plasmid cDNA contained in the fragment of the target gene of the representative samples parallel with the respective standard curve. An efficiency-corrected method has used the calculation of the relative expression level of the fish gonadal genes on different developmental days. The correlation of the standard curve for the gene analysis was -0.999. All samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (gadph; GenBank accession no. EU042107), and the maximum value (control value) of every gene was defined as one. The reaction condition was: 95^0C, 10 minute; 35 cycles, 95^0C, 15 second; 60^0C, 1 minutes. The substance reagent for quantitative analysis was added as follows: SYBR Green I Master Mix (1µl), Gene-specific primer forward (3 µM) (0.2µl), Gene-specific primer reverse (3 µM) (0.2µl), dsH2O (2.6µl), Template (cDNA) (2 µl) (Han *et al*., 2018; Qin *et al*., 2019; Huang *et al*., 2021, 2023; Zhu *et al*., 2022).

2.3 Analysis Data

All data was tabulated in Microsoft Excel 2016 and all statistical analysis was conducted with the SPSS v.22.0 software (SPSS Inc., USA) by one-way ANOVA followed by the Duncan Multiple Range Test (DMRT) and Tukey's test with confidence interval 95%. Comparing data with the differences $p<0.05$ indicates a significant difference in the development expression of the genes in the gonad.

3. Results and Discussion

3.1 Results

In the present experiment, we used under yearling, orange-spotted grouper with MT-oral administration to induce precocious sex change from the immature ovary to develop to be testicular tissue with active spermatogenesis (Table 2). In this present experiment, all the female gonads of 5-aza-treatment had no spermatogenic cells and well-developed oocytes (primary oocytes) as well as the small females in the control fish

set. Thus, the gonads of the non-sex changing fish and the control female fish were considered normal ovaries (Table 2) (Figure 1H-1J).

In the group in which fish were treated with MT feed + 5-aza-induced, ovarian elements were absent and testes were undergoing active spermatogenesis.

Table 2. The sexual phase of the orange-spotted grouper, *Epinephelus coioides* during the experimental period, including three months of MT-oral administration and after one month of MT-termination

Administration periods [treatment (months) + termination (months)]	Sample No.	Total Length (cm)	Body Weight (g)	Sexual phase		
				Female	Interphase	Male
Initial control						
0 d	3	9.00 ± 0.57	10.48 ± 1.58	3	0	0
1 + 0	3	10.40 ± 1.37	19.55 ± 9.00	3	0	0
2 + 0	4	12.90 ± 1.50	34.80 ± 10.00	4	0	0
3 + 1	8	13.78 ± 1.57	40.18 ± 5.14	8	0	0
MT						
1 + 0	3	9.90 ± 0.84	16.47 ± 4.23	0	0	3
2 + 0	4	10.20 ± 0.89	17.07 ± 4.58	0	0	4
3 + 1	6	10.59 ± 1.15	17.54 ± 4.29	0	1	5
5-aza						
1 + 0	3	10.70 ± 1.51	19.97 ± 7.60	3	0	0
2 + 0	4	12.00 ± 1.40	27.60 ± 7.30	4	0	0
3 + 1	6	12.66 ± 1.54	32.80 ± 2.36	6	0	0
MT + 5-aza						
1 + 0	3	9.40 ± 0.77	12.18 ± 3.51	0	0	3
2 + 0	4	10.32 ± 1.02	17.65 ± 4.66	0	0	4
3 + 1	5	11.18 ± 0.63	20.13 ± 2.31	0	2	3

Table 3. The changes in body weight of the orange-spotted grouper, *Epinephelus coioides* during the experimental period, including three months of MT-oral administration and after one month of MT-termination

Treatments	[treatment (months) + termination (months)] Initial Body Weight (g)			
	0	1+0	2+0	3+1
Control	10.48±1.58[a]	19.55±9.00[a]	34.84±10.0[a]	40.18±5.14[a]
MT	10.40±1.58[a]	16.47±4.23[a]	17.07±4.58[c]	17.54±4.29[b]
5-aza	10.48±1.58[a]	19.97±7.60[a]	27.60±7.30[b]	32.80±2.36[a]
MT + 5-aza	10.48±1.58[a]	12.18±3.51[a]	17.65±4.66[c]	20.13±2.31[b]

Various stages of spermatogenic germ cell development from spermatogonia to spermatozoa were seen in the testis (Figure 1K-1L). Within 1 month after the withdrawal of MT treatment, two fish from five fish has a transitive stage of gonads with a view of early germ cells (Table 2) (Figure 1G). The body weight data was accumulated during a period of four months (three months of MT-oral administration and a month after MT-termination) (Table 3). In The average body weight of the fish was obtained from the total weight of the fish in each tank and individual measurements were taken during all subsequent sample periods (Table 3). This study showed that treatment control (40.18 ± 5.14 g) and 5-aza (32.80 ± 2.36 g) resulted the higher of body weight during the treatment (3+1) compared to other treatments. The administration of 5-aza in this study was not able to support the growth of orange-spotted grouper. This result is also similar to the results of other studies that reported treatment with a DNA methyltransferase (5-aza-Dc), surviving fish and body weight (BW) both in males and females is smaller than control (without 5-aza-Dc) (Ribas *et al*., 2017; Li and Liu, 2021).

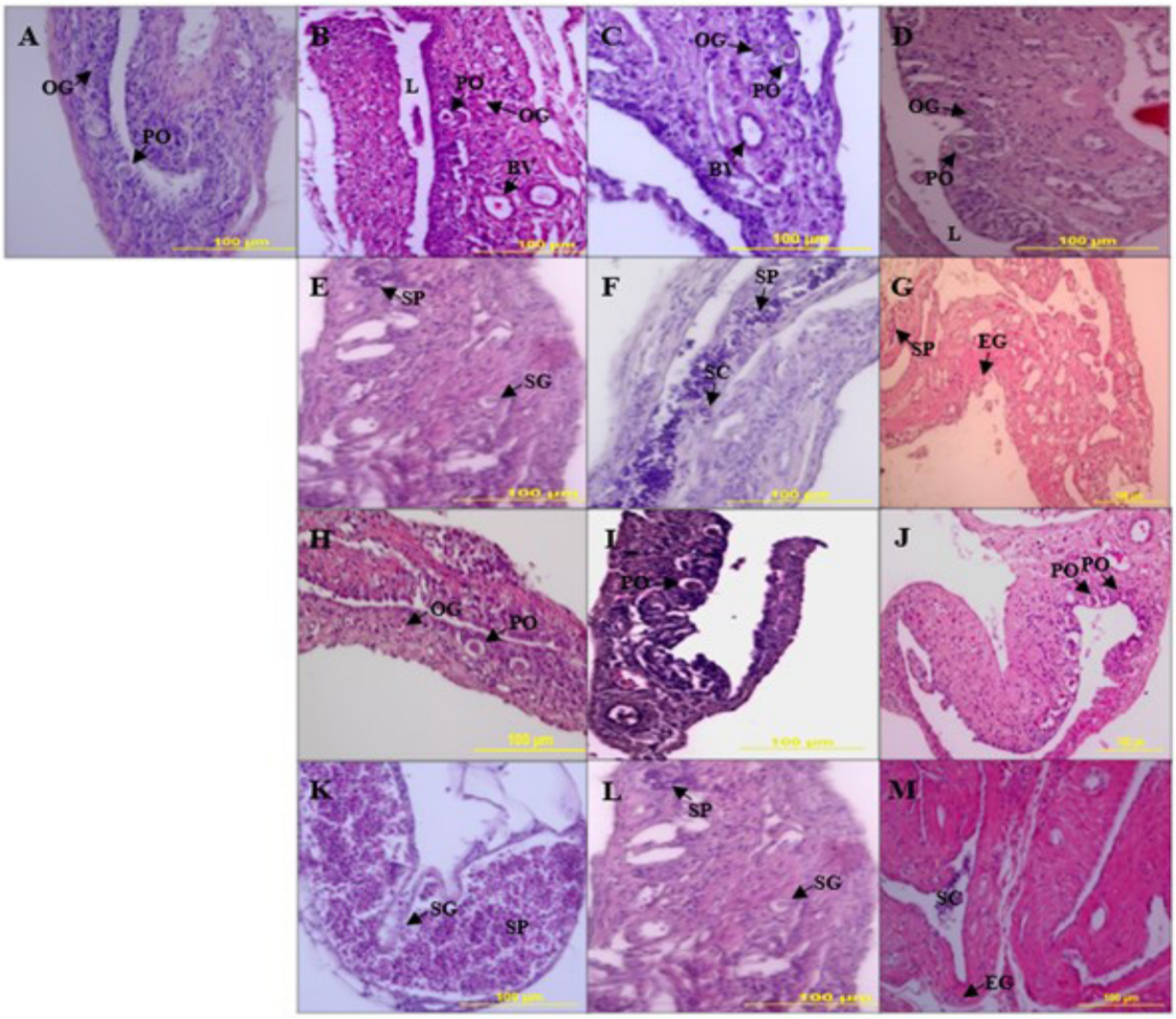

Figure 1. The development of gonads in the protogynous, orange-spotted grouper (A-D) Control group; (E-G) MT-feed group; (H-J) 5-Aza induce group; (K-L) MT Feed+5-Aza induce group. The gonadal morphology of orange-spotted grouper status is shown as follows: (A-D) Initial control female; (A) Initial control female at 0 days; (B-D) Initial control female at one-, two-, and three-months respectively; (E-F) MT-induced male with active spermatogenesis one and two months after MT-oral administration; (G) Regenerated female after a month of MT-termination; (H-J) 5-Aza treatment at one, two, and three months respectively; (K-L) MT + 5-Aza induced male with active spermatogenesis one and two months after MT-oral administration; (M) Regenerated female after one month of MT-termination. L, central lumen; OG, oogonia; PO, primary oocyte; RO, regressed oocyte; SG, spermatogonia; SC, spermatocyte; SP, spermatozoa; EG, early germ cell.

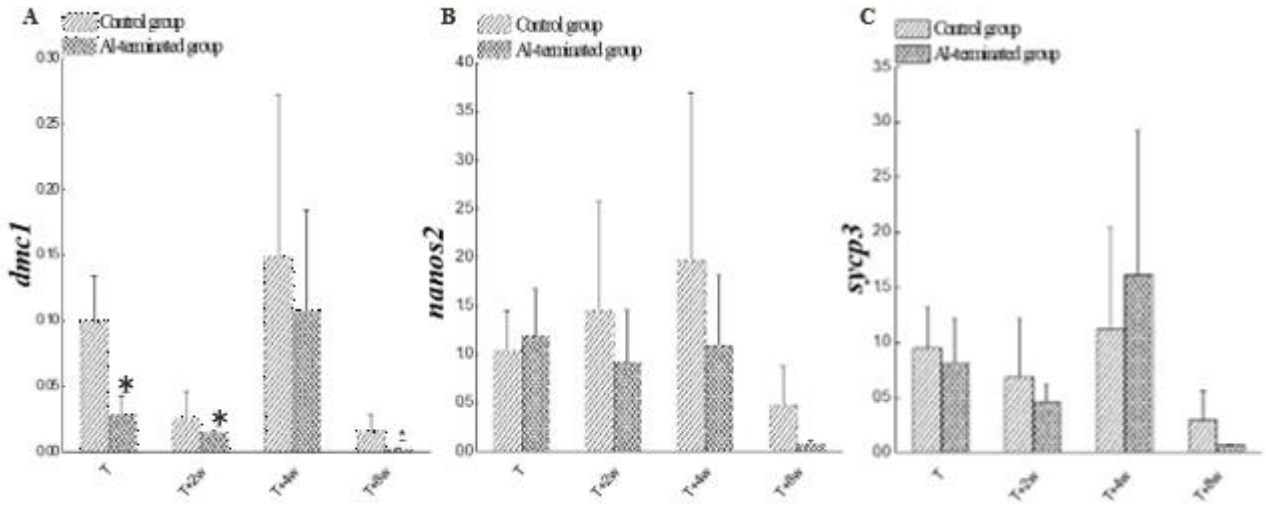

Figure 2. The AI-treated fish were functional males after three months of oral administration (T). The AI-induced maleness was reversible. (A), (B), and (C) expression of dmc1, nanos2, and sycp3 genes, respectively, during the male-to-female sex change that occurred after AI termination. Superscript letters indicate one-way ANOVA and a Duncan test ($P < 0.05$). Asterisk indicates an independent sample t-test ($P < 0.05$).

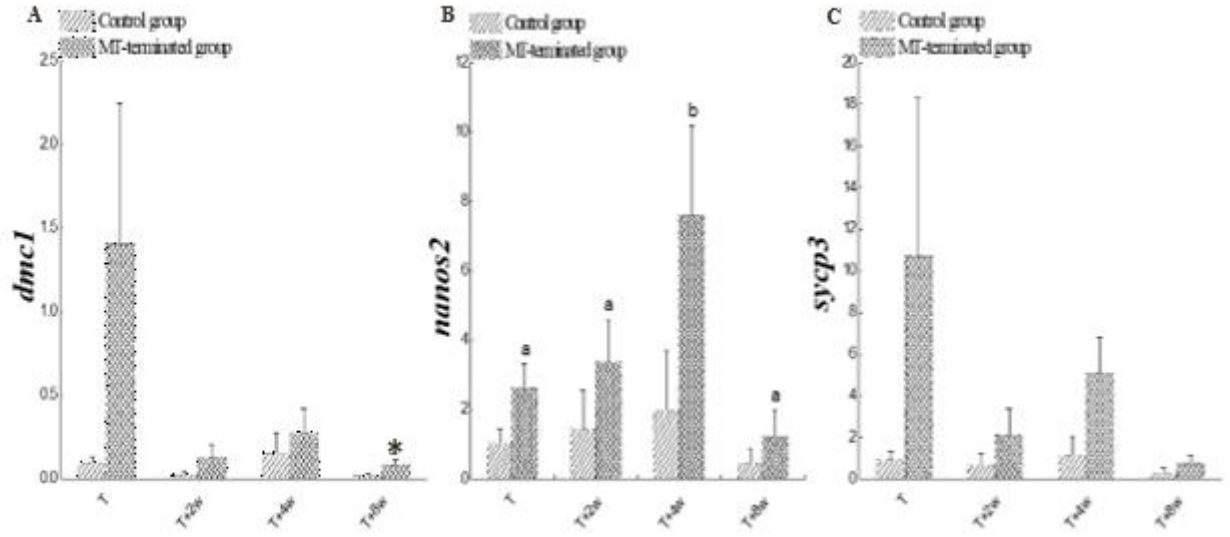

Figure 3. The MT-treated fish were functional males after three months of oral administration (T). The MT-induced maleness was reversible. (A), (B), and (C) expression of dmc1, nanos2, and sycp3 genes, respectively, during the male-to-female sex change that occurred after MT termination. Superscript letters indicate one-way ANOVA and a Duncan test ($P < 0.05$). Asterisk indicates an independent sample t-test ($P < 0.05$).

This study suspect that the use of 5-aza can play a role in sex-change in orange-spotted grouper fish, but its use actually reduces fish growth. Moreover, we suspect that there is an effect on the energy and absorption of nutrients in the fish body, as well as the energy derived from protein, fat and carbohydrates which should be used by fish for growth is mostly used to respond to 5-aza, especially in the process of sex-change. There were no significantly different between body weight at the onset of the experiment (day 0) and a month during the treatment (1+0) in all treatments. The body weight of control fish and fish that were treated with 5-aza alone was higher than the body weight of those fish that were treated by MT alone and MT+5-aza after three months of treatment and one month after termination (3+1). The treatment groups which received 50 mg/kg of MT were growing lower in weight than the controls throughout the study. There were significant differences ($p<0.05$) found in the body weight rate between fish with MT treated with the initial control fish. All juvenile fish in the initial control group had immature ovaries consisting of the ovarian cavity and a few oogonia (Figure 1A). The result showed that the initial body weight on the one-month treatment (1+0) there was no significant difference ($p>0.05$) between all treatments. Meanwhile, on the two-month (2+0) treatment the control showed the highest initial body weight 34.84±10.0 g and significant difference ($p<0.05$) compared to all treatments. Moreover, on the three-month (3+0) treatment, there was increase in the initial body weight in all treatments. Control treatment 40.18±5.14 g and 5-aza (32.80±2.36 g) had higher initial body weight and significant difference ($p<0.05$) compared to MT and MT+5-aza treatment. The blood vessel organization was observed at the ovary (Figure 1B-1C). As the experiment progressed, the immature ovaries of all control fish developed slowly and oogonia and a few primary oocytes were detected in the ovary at the end of the experiment (Figure 1B-1D).

In the AI-oral administration, the qPCR confirmed that three months after AI termination, dmc1 genes showed significantly lower expression compared to the control fish (females) in 0 days, two weeks, and eight weeks (Figure 2A). In contrast, there were no differences were found in the nanos2 and sycp3 genes compared to the control fish (normal ovary) after two, four, and eight weeks AI termination (Figure 2B - 2C).

The AI and MT-treated fish were functional males after three months of oral administration (T). The AI-induced maleness was reversible. (A), (B), and (C) expression of dmc1, nanos2, and sycp3 genes, respectively, during the male-to-female sex change that occurred after AI termination. Different superscript letters indicates the significance different between treatments (Figure 2 and Figure 3).

3.2 Discussion

We showed that one month after treatment with MT-oral administration, the female gonad in grouper had a few advanced male germ cells and thus indicating the onset of spermatogenesis. This result revealed that this fish's bipotential and sex change could be manipulated by the oral administration of androgens. It is well known that exogenous androgen, induced the masculinization of genetic females (Nakamura *et al*., 2003). Androgens may suppress gonadal aromatase activity and ovarian growth, and also stimulate the development of male germ cells (Nakamura *et al*., 2003).

The importance of this study was to induce a reversible sex change in the under-yearling of orange-spotted grouper by applying exogenous MT with oral administration. Here, we used histological data on testicular differentiation and ovarian development of orange-spotted grouper. The histological characteristics of gonads during sex-change have been reported for some fishes (i.e. *Thalassoma duperrey*; *Epinephelus rivulatus*) (Nakamura *et al*., 2003). This result is in agreement with results reported previously on this grouper by (Nakamura *et al*., 2003). MT is also effective at inducing sex-change in some sequential hermaphroditic fishes, such as *Epinephellus tauvina* (Chen *et al*., 1977), *Epinephellus fario* (Kuo *et al*., 1988), *Epinephellus bruneus* (Oh *et al*., 2013), *Epinephelus malabaricus* (Murata *et al*., 2014), and sea bass, *Dicentrarchus labrax* L. (Blázquez *et al*., 1995).

In the protogynous orange-spotted grouper, Cyp19a1a protein or mRNA expression in the follicle layer cells in the gonad was dropped significantly during artificial permanent sex reversal from female to male induced by MT treatment (Zhang *et al*., 2004). Cyp19 encodes for a steroidogenic enzyme, aromatase, which is responsible for catalyzing the aromatization of androgens to estrogens and this is a key gene in ovarian differentiation in all species of teleosts examined (Kitano *et al*., 2000). This finding suggests that when the Cyp19a1a protein levels fall below a physiological threshold, oocytes no longer survive, and the spontaneous androgen levels can initiate spermatogonial proliferation in the ovary. However, one month after the withdrawal of MT-oral administration, the sex of the juvenile grouper was returned to femaleness even though the fish have undergone MT-induced masculinization. This outcome demonstrates precocious sex-change from under yearling, orange-spotted grouper utilizing oral MT treatment is impermanent. In protogynous fish, the ste-

roid-induced males are transient and reversible in sequential sex-change (Nakamura *et al*., 2003). Moreover, the normal time for female-to-male sex change of this fish is around 4-5 years depending on the environment, hormone, and nutritional condition in this fish. The oocyte development in orange-spotted grouper begins at the age of 1-3 years (Wang *et al*., 2017) and then the gonads will mature as females at around 4-5 years. After maturing female gonads, this fish will change sex from female to male (Chen *et al*., 1977; Heemstra and Randall, 1993; Quinitio *et al*., 1997). The roles of androgens in fish sexual differentiation are disconcerting (Bhandari *et al*., 2006). Treatment of protogynous hermaphrodites with androgens (i.e. 11-ketotestosterone, or the synthetic androgen MT) can masculinize and enhance sex reversal in *Sparisoma viride* (Cardwell and Liley, 1991), as well as groupers *Epinephelus akaara* (Li *et al*., 2006), *Epinephelus suilus* (Tan-Fermin *et al*., 1994), and *Epinephelus bruneus* (Oh *et al*., 2013). Withdrawal of MT treatment in this latter species results in reversion back to an ovarian condition, similar to the effect described above for estrogen withdrawal and transient sex reversal in *Acanthopagrus schlegeli* (Tan-Fermin, 1992).

Treatment of female *Coryphopterus nicholsii*, with androgens or an aromatase inhibitor, were both capable of causing sex reversal (Kroon and Liley, 2000), indicating the importance of androgens, and possibly lack of estrogens, on sex differentiation in this species, but the gonadal transformation is not always complete with these hormone treatments: In *Thalassoma bifasciatum* implanted with testosterone (Kramer *et al*., 1988), ovarian degeneration occurred without testicular development, however transformation of the blue head color (a secondary sex character) was complete.

MT treatment could induce sex change, but the results showed that it is unstable sex change and this indicates that fish reverted to females after MT termination and maintenance of secondary sex determination as well as its correlated with sex steroids. In protogynous fish, gonadotropin or gonadotropin-releasing hormones (GnRH) are also able to induce sex reversals, resulting the development of germ cells and somatic cells in gonad. GnRH is the pituitary gland in endocrine system to stimulate the production of follicle-stimulating hormone and luteinizing hormone. These gonadotropins make the sex hormones testosterone, estrogen and progresteron. In *Monopterus albus,* treatment with a GnRH analog from salmon fish elevated androgen levels and induced functional sex reversals (Tao *et al*., 1993). Peptides regulating gonadotropin production such as GnRH analogs also can strongly influence sex reversal in *Thalassoma bifasciatum* (Kramer *et al*., 1988). In *Thalassoma bifasciatum*, human chorionic gonadotropin (hCG) injection can rapidly induce the appearance of testicular tissue in females within six weeks in most animals (Koulish and Kramer, 1989). The hCG hormone has roles in inducing maturation, ovulation and fish spawning. This related with the results that the involvement of ypophyseal and pituitary levels control in mediating environmental cues for sex change in orange-spotted grouper.

The results showed that the AI-oral administration, the qPCR confirmed that three months after AI termination, dmc1 genes showed significantly lower expression compared to the control fish (females) in 0 days, two , and eight weeks (Figure 2A). In contrast, there were no differences were found in the nanos2 and sycp3 genes compared to the control fish (normal ovary) after two, four, and eight weeks of AI termination (Figure 2B-2C). During MT-induced precocious sex-change of the fish, the expression levels of dmc1 genes in the gonad were increased in the MT-treated fish, and the differences were significant after 8 weeks of treatment termination compared to the control fish (Figure 3A). The expression of nanos2 was identified in fish that displayed maleness (MT-administered fish) present no difference between 0 days after termination, two and eight weeks. The higher expression of genes showed at four weeks (Figure 3B). There were no differences performed on the sycp3 gene in the MT-treated fish after three months of oral administration, two, four, and eight weeks after termination (Figure 3C).

In the present study, we demonstrate the dmc1, nanos2, and sycp3 gene expression. Our result revealed that during AI-induced sex-change of the orange-spotted grouper, dmc1 genes showed significantly lower expression compared to the control fish. Conversely, the expression levels of dmc1 genes in the gonad were increased in the MT-treated fish, and the differences were significant 8 weeks after treatment termination compared to the control fish. The expression analysis of dmc1 has been shown in Japanese eels, *Anguilla japonica*. In this species, the dmc1 protein is expressed in the meiotic prophase specifically during spermatogenesis (Kajiura-Kobayashi *et al*., 2005). Further examination revealed that dmc1 was localized only in early primary spermatocytes, but not in late primary spermatocytes, suggesting that the expression of dmc1 is limited to the early primary spermatocytes and that dmc1, thus, is a highly appropriate molecular marker for identifying the entry of spermatogonia into meiosis (Kajiura-Kobayashi *et al*., 2005).

The present study had shown that dmc1 protein expression in grouper is only expressed in the ear-

ly meiotic prophase and could be an ideal molecular marker for the entry of germ cells into meiosis in the orange-spotted grouper testis. The expression level of nanos2 and sycp3 genes showed no significantly different expression in the AI-induced. On the other hand, in the MT-treated fish, the higher expression of nanos2 genes showed at four weeks after termination.

Nanos2, known markers of the early and also expressed preferentially in the spermatogonia fraction isolated from immature and from adult rainbow trout and are downregulated after spermatogonia differentiation (Bellaiche *et al*., 2014). In drosophila, nanos are required for the maintenance of female germline stem cells and hence the continuous production of oocytes in adults (Forbes and Lehmann, 1998). Similarly, conditional knock-out of mouse nanos2 leads to the loss of spermatogonial stem cells in the adult testis (Sada *et al*., 2009). Furthermore, overexpression of nanos in the female drosophila germline or nanos2 in the mouse testis is sufficient to lock early germ cells into a stem cell fate (Roeder, 1997).

The synaptonemal complex (sycp) has been observed in meiotic cells in most sexually reproducing organisms (Roeder, 1997) and the synaptonemal complex protein 3 (known as sycp3) are components of the axial/ lateral elements of the synaptonemal complex in male germ cells (Aarabi *et al*., 2006). Sycp3 is assumed to constitute the core of the lateral element complex and function as a molecular framework to which other proteins attach. It has a role in regulating DNA binding to the chromatid axis, sister chromatid cohesion, synapsis, and recombination (Parra *et al*., 2004). As in the case of mammals, in the medaka fish, *Oryzias latipes*, sycp3 is expressed solely in meiotically dividing cells. Since the antibody against medaka, sycp3 is cross-reactive to other fishes, it should be generally useful as a meiosis-specific marker in fish germ cells (Iwai *et al*., 2006). In this present study, our results demonstrate that the dmc1, nanos2, and sycp3 expression are gradually reduced after AI termination, conversely, dmc1, nanos2, and sycp3 were gradually increased after MT-termination and these revealed that AI/MT-induced masculinization is impermanent.

Fish were treated with MT and 5-aza-induced, ovarian elements were absent and testes were undergoing active spermatogenesis. This study showed that treatment control and 5-aza resulted the higher of body weight during the treatment (3+1) compared to other treatments. The treatment with 50 mg/kg of MT was growing lower in body weight than the control. In this study, MT has impact on fish growth with the dose of MT 50 mg/kg. Several studies reported that the administration of MT with the dose of 5.0, 10.0 and 20.0 mg/kg resulted the best growth occurring at the highest dosage in *C. striatus* (Nirmala and Pandian, 1983). The administration of MT in *Penaeus indicus* with the dose of 2.5 mg/kg diet induced growth (Vatheeswaran and Ali, 1986). Several studies reported that MT induced appatite and enhance food consumption in *Carassius auratus* (Yamazaki, 1976) and *C. punctatus* (Muniasamy *et al*., 2019). The dosage of 100 mg/kg and 60 mg/kg MT is the optimal dosage for these fresh water fishes *C. mrigala*. Meanwhile in this study, the administration of MT resulted the lower body weight. There are indicates that the administration of MT to the hermaprodite fish is different with non-hermaprodite fish. This results also similar with several research that the higher dosage a few steroids (MT) exhibited some harmful effects (Donaldson *et al*., 1979) and depressed food consumption in *C. striatus.*

4. Conclusion

In this study, MT treatment could induce sex-change, but it is unstable changes because fish reverted to female after MT termination and maintenance of secondary sex determination is correlated with sex steroid. The administration of 5-aza could not promote ovarian differentiation in the sex-changing fish via other mediators in the gonadal differentiation signaling pathways, due to lower doses. The dmc1 protein expression could be an ideal molecular marker for the entry of germ cells into meiosis in the orange-spotted grouper testis. The expression level of nanos2 and sycp3 genes showed no difference in the AI-induced, it is assumed that the lack of germline stem cells in the orange-spotted grouper ovary is consistent with the absence of nanos2 expression in germ cells of the ovary.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author is as follows, OCA; discussed and critically revised the article. AIS; collected and analyzed data, discussed, and prepared the draft. CFC and GCW; contributed to the main idea

of research and critically revised the article. MUS; analyzed data, discussed, and searched for related papers. DER; searched for related paper and layouted paper. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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