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ORIGINAL RESEARCH

Effect of synthetic androgen treatment on the gonad of juvenile longtooth grouper (*Epinephelus bruneus*)

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Abstract Commercially significant groupers are protogynous hermaphrodites. Since their sex change from female (ovary) to male (testis) occurred in a larger size and older age, capturing or maintaining males in the aquaculture farm is very difficult. In the present study, juvenile longthooth groupers (2^+ years old) were implanted with two doses of 17alpha-methyltestosterone (MT) cholesterol pellet (2 mg or 5 mg MT kg^{-1} BW) for the induction of artificial sex reversal. Individuals were sampled at 30 and 80 days after treatment. All the control group fish had immature ovaries contained with primary oocytes and germ cells. Fish treated with MT for 30 days had sex-transitional gonad consisted of few primary oocytes and testicular tissues. By using immunohistochemistry, we identified the proliferating and apoptotic cells in the gonad of this sexual stage. After 80 days for MT treatment, gonads of all individuals were filled with germ cells at all stages of spermatogenesis, and the newly formed efferent duct contained active spermatozoa. Since sperm motility was confirmed, MT treatment successfully induced functional sex reversal in pre-pubertal longtooth grouper. This method may dramatically reduce the cost and time required for the production of longtooth grouper.

Keywords Sex reversal . Sex change . Ovary . Testis . 17alpha-methyltestosterone . Grouper . Androgen

Introduction

Groupers (Family Serranidae: subfamily Epinephelus) are distributed in tropical and subtropical waters in the world (Nakamura et al. 2005). Groupers are commercially and recreationally valuable fish (Rimmer and Glamuzina 2019). In recent years, due to overfishing and habitat degradation, groupers' population size has been decreasing (Morris et al. 2000). Therefore, grouper aquaculture has been conducted intensively for stable supply in many areas of the world, especially in Asia countries (Pierre et al. 2008; Rimmer and Glamuzina 2019).

Almost all groupers are known as protogynous hermaphrodites (Devlin and Nagahama 2002; Nakamura et al. 2005; Kobayashi et al. 2013a). They mature first as females and much later change into males depending on their body size and age or complex social structure (Mackie 2003; Nakamura et al. 2007; Chen et al. 2020). This kind of reproductive strategy raises two significant problems for the management of grouper aquaculture (Debas et al. 1990). Firstly, the sex ratio in the wild is biased toward a female, making it challenging to collect mature males stably. Secondly, it is difficult to maintain large individuals in captivity for an extended period until they grow to an appropriate size for a natural sex change. To solve the mature male's unavailability in broodstock, techniques of artificial induction of the sex reversal

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from reproductively mature females into males treated with androgens or an aromatase inhibitor (AI) have been developed for various grouper species in the past decade (Kuo et al. 1988; Nakamura et al. 2005; Bhandari et al. 2006; Kobayashi et al. 2013a; Budd et al. 2015). In recent years, this technique has also been attempted to prepubertal juvenile fish to produce the smaller male in several grouper species (Tan-Fermin et al. 1994; Sarter et al. 2006; Murata et al. 2014; Lee et al. 2014; Rodrigues-Filho et al. 2020). In these attempts, spermatogenesis was successfully induced in the juvenile's gonads, but the current protocols are poorly reproducible, and the characteristics and functionalities of sex-reversed small males have not been clarified.

The longtooth grouper, *Epinephelus bruneus*, is one of the important aquaculture species in Asia, including Japan (Inoue et al. 2016). In our breeding farm, the first sexual maturation of females occurs when the individual about 5 kg in body weight (at eight years old), while natural sex change occurs from 10 years old and 7-8 kg in body weight. The growth of longtooth grouper is slower than other grouper species, and growth stagnation at low temperatures (less than 18-20 °C) in winter has occurred (Inoue et al. 2015). Thus, it takes at least three years for cultured longtooth groupers to attain marketable size in Japan. Therefore, aquaculture farmers required the high growth strain larvae of longtooth grouper.

The aim of this study was to elucidate the functionality of sex-reversed small males for grouper breeding. As a first step, we induced female-to-male sex reversal in juvenile longtooth groupers by synthetic androgen (17α-methyltestosterone: MT). Additionally, the effect of MT on the gonads was observed histologically. Besides, the immunostaining of proliferating and apoptotic cells in the gonads was carried out following the sex reversal process. Finally, we confirmed the spermiation and sperm motility of individuals after 80 days MT treatment. The results generated in this study contribute to the grouper aquaculture.

Materials and methods

Experimental Fish

The juvenile longtooth grouper (2-year-old) reared in Aquaculture Research Institute of Kindai University were used in this study. The experimental fish had a wide variation in body weight (BW: 166.9 ± 56.2 g). All experimental fishes were kept in the running seawater tank under the natural condition (sea water temperature ranged from 19.5 to 26.5 °C). And fishes were tagged for identification with pit tag (Tanaka Sanjiro Co., Ltd. Fukuoka, Japan).

Hormone treatment

The androgen pellets were produced according to the previous report (Lee et al. 1986). The synthetic androgen, 17α-Methyltestosterone (MT: Sigma-Aldrich, St. Louis, MO), was dissolved in ethanol and mixed with cholesterol powder. The mixture was dried, compounded with molten cocoa butter, and then compressed into pellets with mold. Pellets with two different MT concentrations were prepared individually according to the body weight of each fish. On May 5, 2019, after anaesthetization with FA-100 (DS Pharma Animal Health Co., Ltd. Osaka, Japan), MT pellets were implanted into the fish's dorsal muscle. Each fish received no pellet (control group: 17 fish); a pellet that had a 2 mg MT kg^{-1} BW (MT-low: 16 fish); a pellet that had a 5 mg MT kg^{-1} BW (MT-high: 19 fish).

Sampling

Experimental fish were checked for identification with a tag-reader before sampling. At 30 and 80 days after hormone treatment, fish were sampled after being euthanized by decapitation. The gonads of experimental fish were dissected and rinsed with 0.1M PBS. After measuring gonad weight (GW), half of the gonad was fixed with Bouin's fluid for histological observation. The gonad somatic index (GSI) was calculated by GW / BW x 100.

All experimental procedures conformed to "Regulations for Animal Experiments and Related Activities at Kindai University" and were reviewed by the Animal Experiment Committee of Kindai University, and

finally approved by the university's President.

Confirmation of male function

In order to confirm male function, spermiation and sperm motility of the MT-treated fish were examined. Briefly, two days before the last sampling (80 days after MT treatment), human chorionic gonadotropin (HCG, Aska Animal Health Co., Ltd. Tokyo Japan) was injected to facilitate spermiation. The HCG dissolved in 0.6% NaCl solution was injected into the dorsal muscle (500 IU kg/1 BW). Before decapitation at the last sampling, spermiation was identified by milt flowed out from the genital pore when the abdomen was gently massaged. The sperm motility of all milt samples was checked by microscope. In addition, gonads were removed from four randomly selected fishes from MT treatment groups whose spermiation could not be confirmed. The removed half of gonads in these fishes were dissected in artificial seminal plasma (NaCl 135 mM, KCl_2 mM, $MgCl_2$ 2.3 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 20 mM, 20 mM buffered with HEPES-NaOH at pH 8.2) and filtered to remove debris. Then 0.2 ml of the supernatant was dropped onto a glass slide and diluted with an equal volume of seawater. Immediately after dilution, the sperm motility was observed under a microscope.

Histological observation

To analyze the gonadal sexual stage, bouin's fixed samples from each individual were dehydrated in a series of alcohol, clarified in ClearPlus (FALMA, Tokyo, Japan), and then embedded in paraffin. Seven-micrometer-thick tissue sections were collected from paraffin blocks. After dewaxing and hydration, the sections were stained with hematoxylin and eosin.

To identify the proliferating and apoptotic cells in the gonads, immunohistochemical (IHC) staining was performed. According to the manufacturer's instructions, detection and visualization of the primary antibodies were accomplished using ImmPRESS reagent (Vector laboratories). Briefly, paraffin sections of the gonad, after deparaffinization and dehydration, were rinsed in TTBS. They were treated with 0.3% H_2O_2 for 15 min to block endogenous peroxidase. Following incubation with 10% normal horse serum for 15 min, the sections were incubated with primary antibodies against Proliferating Cell Nuclear Antigen (PCNA, 1:1000 dilution; product no. ab92552. Abcam, Cambridge, MA, USA) or active caspase-3 (1:200 dilution; product no. bs-0081R, Bios, MA, USA) that is one of the key enzymes for cell apoptosis. After 2 hours of incubation at room temperature, sections were incubated with a secondary antibody. After washing with TTBS, the primary antibody signals were visualized using 3, 3' -diaminobenzidine (Wako chemical, JAPAN).

Results

Gonadal histology

All gonads examined in this study were about 2-5 mm diameter, elongated, and flattened (Fig. 1). Blood vessels (BV) were located on the tunica gonad of the dorsal side. Based on the histological observation, we classified the gonadal sexual stages of individual fish into three stages, as follows:

Ovary: Typical ovarian cavity (OC) was located in the center of the gonad. A large number of oocytes at perinucleous stage and primary germ cells were present in the gonads (Fig. 1A). To confirm proliferating and apoptotic cells in the gonad, IHC was performed. Positive signals for PCNA were seen in primary germ cells (Fig. 2A), but caspase-3 immunoreactivity was not seen in the gonad (Fig. 2B).

Sex-transitional: Spermatogonial cells (spermatocyte and spermatid) and primary germ cells, which had PCNA-immunopositive signals (Fig. 2C), were dominant constituents in the gonad (Fig. 1B). Simultaneously, few oocytes were present in the gonad (Fig. 1B). The caspase-3 immunopositive signals were observed in the nucleus of this oocyte (Fig. 2D). The efferent duct was newly formed at the dorsal region of the gonad (Fig. 1B).

Testis: Active spermatogenesis and no ovarian tissues were observed in the gonad (Fig. 1C). The

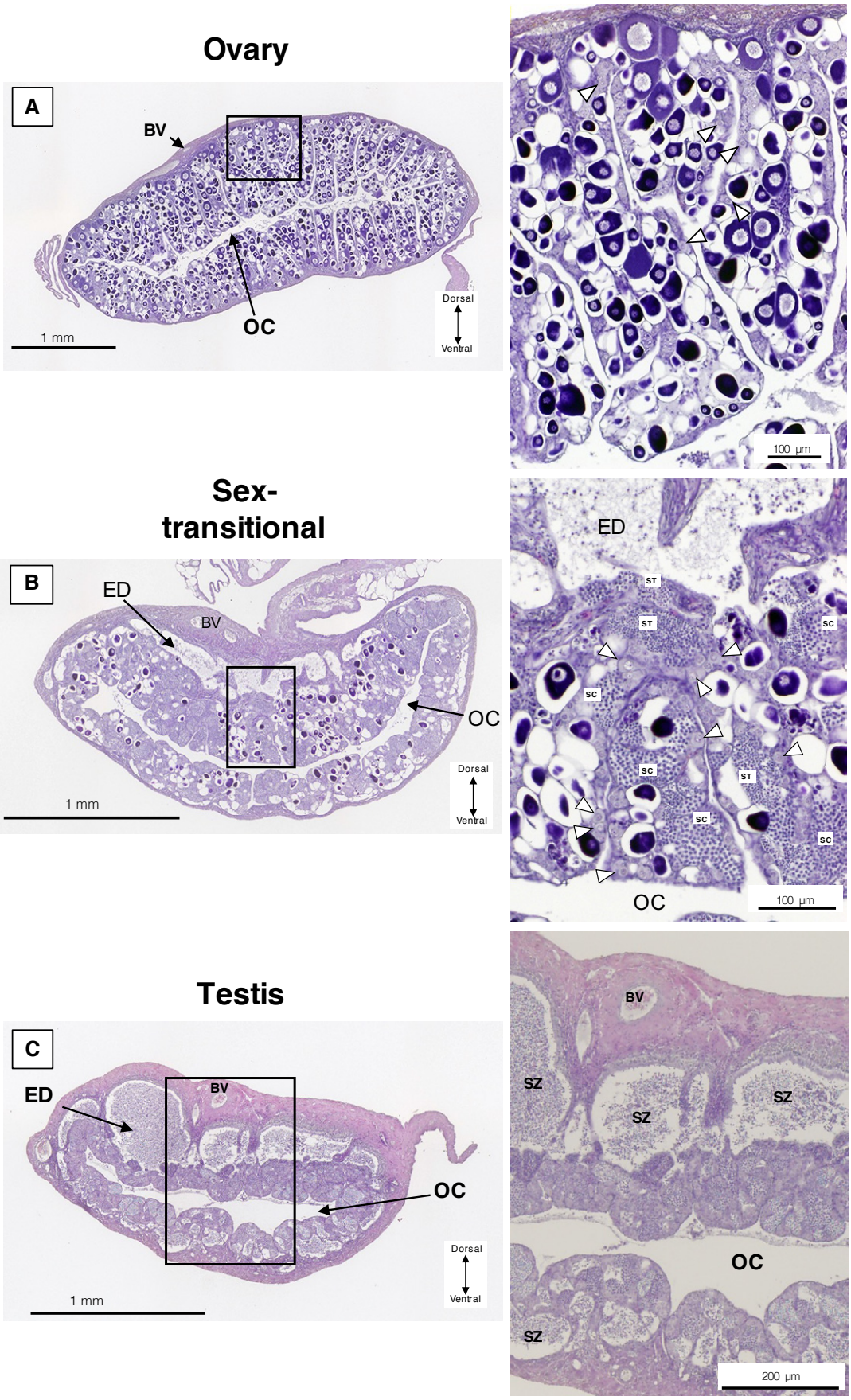

Fig. 1 Representative photomicrographs of the ovary (A), sex-transitional gonad (B), and testis (C) of juvenile longtooth grouper. Sections are stained with hematoxylin and eosin. Arrowheads indicate germ cells. BV, blood vessel; OC, ovarian cavity; ED, efferent duct; SC, spermatocyte; ST, spermatid; SZ, spermatozoa.

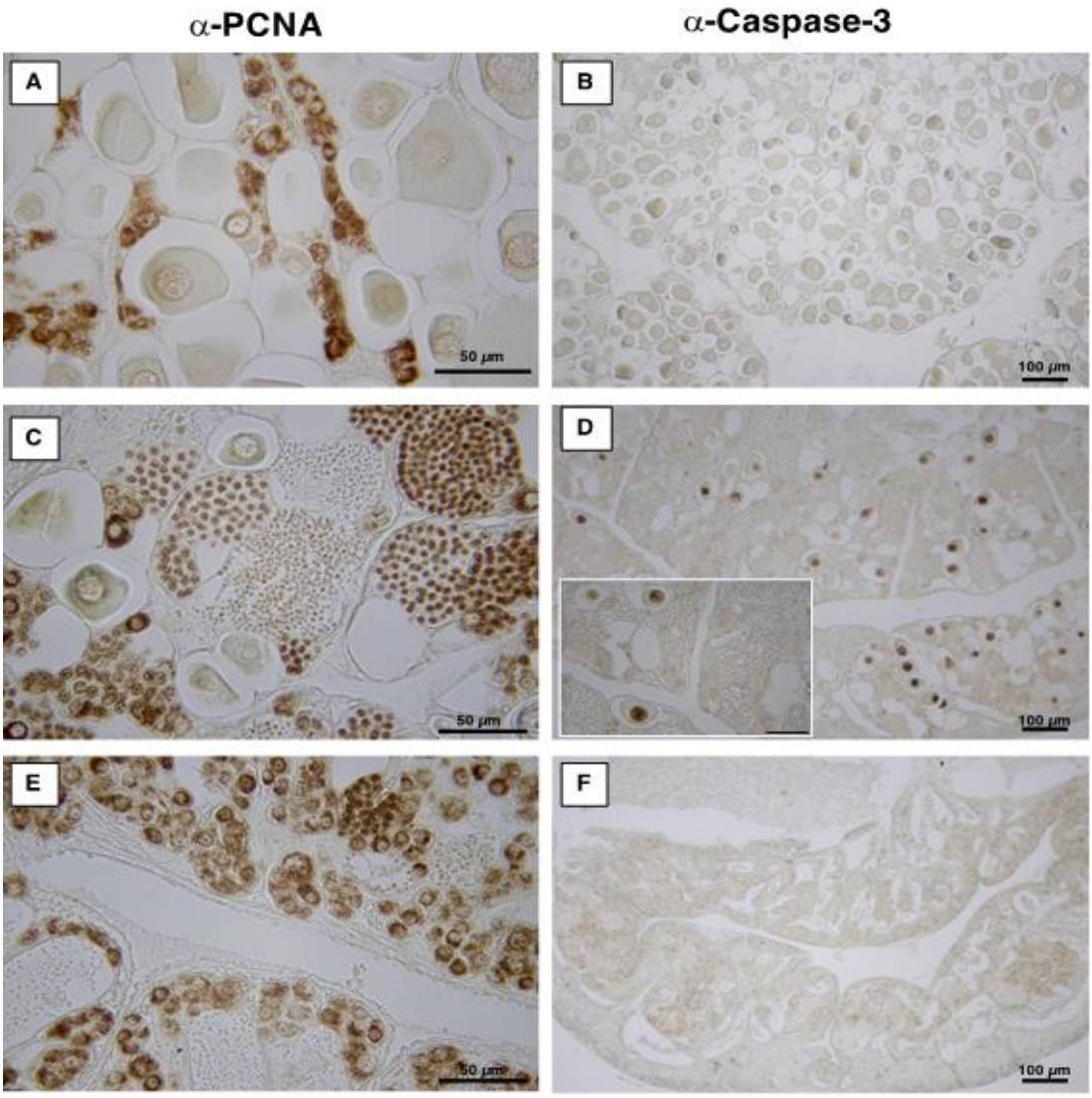

Fig. 2 Localization of PCNA-immunopositive cells (proliferating) and active caspase 3 immunopositive cells (apoptotic) in the different sexual gonads of juvenile longthooth grouper. A, B: ovary (control). C, D: sex transitional gonad (30 days after MT treatment). E, F: testis (80 days after treatment).

efferent ducts were filled with spermatozoa. As a result of immunostaining, PCNA-immunopositive signals were found in germ cells on the germinal epithelium and spermatocytes (Fig. 2E), whereas caspase 3 immunopositive signals were not observed (Fig. 2F).

Effect of MT treatment on the gonad of juvenile longtooth grouper

The gonadosomatic index of the experimental fish is shown in Fig. 3. No significant difference was found in either group. During the experimental period, there was no increase in all fish's body weight. Final BW of experimental fishes is 165.2 ± 56 g.

The degree of gonadal sex ratio in each treatment is shown in Fig. 4. In the control group, the gonads of all individuals were ovary. The gonadal sex of all fish after 30 days MT treatment was sex transitional, regardless of the body size of individuals. After 80 days of MT treatment, all fish had fully transformed into the testis. There was no difference between MT doses in the induction ratio of gonadal sex reversal.

After 80 days of MT treatment, we checked the functionality of sex-reversed males. Spermination was observed in 1 out of 11 fishes in the MT-low group and in 1 out of 13 fishes in the MT-high group.

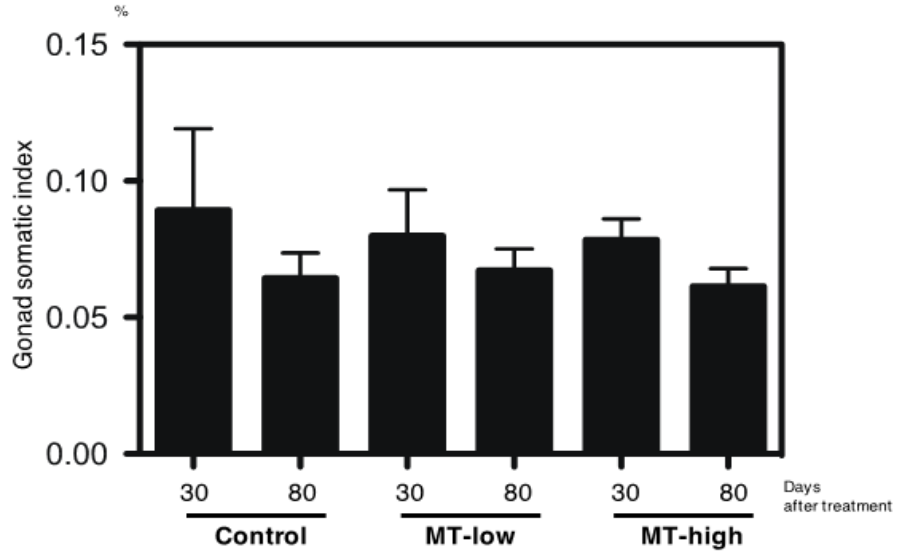

Fig. 3 Effect of MT on GSI of juvenile longtooth grouper. Data are means and error bars represent standard errors. There are no significant differences between the groups (Tukey–Kramer method).

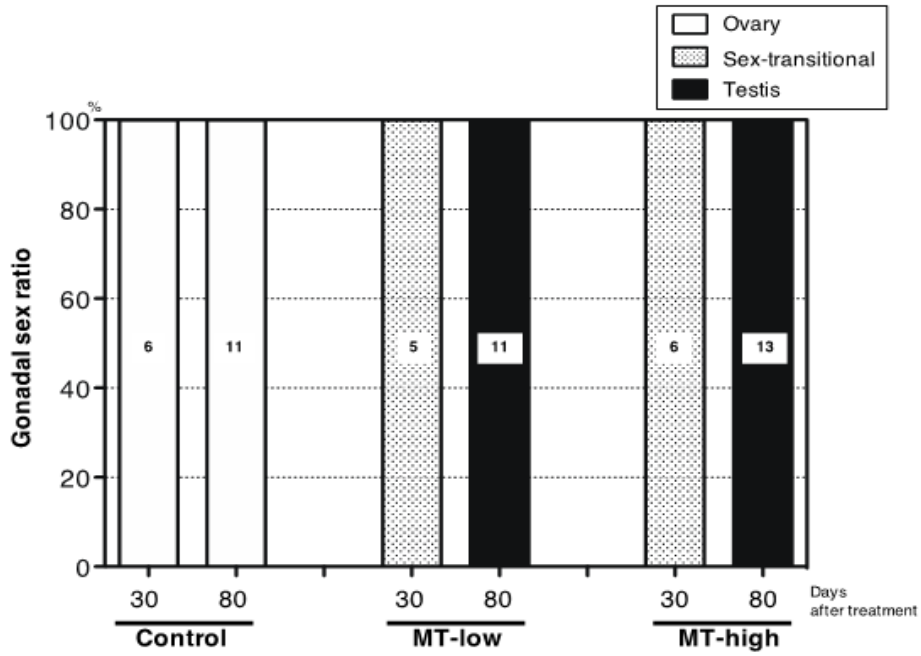

Fig. 4 The gonadal sex ratio of juvenile longtooth grouper treatment with MT. Gonadal sexes were classified into three stages (ovary, sex-transitional, testis). Numbers in bars indicate the total number of fish

Table 1 Male functionality of fish treated with MT for 80 days

Group	*n*	Number of spermiation fish	Number of fishes used for sperm extraction	Number of fishes With sperm motility for at least 15 min.
Control	11	0	0	-
MT-Low (2 mg MT/kg BW)	11	1	4	4
MT-High (5 mg MT/kg BW)	13	1	4	4

The sperm extracted from the gonads of the MT-treated fishes that did not sperminate showed sperm motility for more than 15 minutes in all the animals (Table 1).

Discussion

In this study, we found that the complete sex reversal from immature female to mature male can be induced in juvenile longtooth grouper of any body size by treatment with MT. There were no dose dependencies of MT for GSI, and induction rate of gonadal sex reversal. Remarkably, all of the fish treated with MT in this study underwent complete sex reversal to male. This induction rate of sex reversal (100%) was extremely high compared to other grouper species (e.g., *E. tukura*; 66.7 %, *E. salmonoides*; 87-100%) (Yeh et al.2003). Furthermore, the 80 days MT treatment, together with HCG injection, induced a low amount of milt release. Fertilizing capacity of the sperm in the obtained milt was not confirmed in this study, but active sperm motility was observed. Since more than 50% of the individuals in MT-low group had milt, the 2 mg MT kg^{-1} BW treatment might be sufficient for the induction of sex reversal in juvenile longtooth grouper.

Histological analysis revealed that processes of sex reversal in the gonad of juvenile longtooth grouper. Formation of efferent ducts and proliferation of testicular cells were observed in the gonads 30 days after MT treatment. Simultaneously, caspase-3 immunopositive signals were observed only in the nuclei of primary oocytes, suggesting that these oocytes are responsible for apoptosis. Ovarian tissue and apoptosis cells were not observed in the gonads 80 days after MT treatment. An increase in PCNA positive germ cells and testicular cells was observed in this gonad. These histological changes were similar to the adult natural sex change of other grouper species (Bhandari et al. 2003; Alam and Nakamura 2007; Peng et al. 2020). Natural gonadal sex change in groupers was involved in balancing endogenous estrogens and androgens, which is the most downstream factor in steroidogenesis (Frisch 2004; Nakamura et al. 2005, 2007; Peng et al. 2020). Thus, both treatments of exogenous androgen or an aromatase inhibitor, which is the drug for inhibition of endogenous estrogen synthesis, are adequate for the induction of sex reversal in adult groupers (Kuo et al. 1988; Yeh et al. 2003; Bhandari et al. 2004). Several studies had indicated that androgen treatment could suppress P450 aromatase, which is a critical enzyme for estrogen production, and the subsequent decrease of estrogen is lead to female-to-male sex change (Bhandari et al. 2004; Kobayashi et al. 2013b). However, the levels of endogenous estrogen and androgen of individuals were shallow in this study. Besides, there were tiny immunoreactive signals for P450 cholesterol side-chain cleavage enzyme, which is the critical enzyme for steroid production, in the gonads (Kobayashi et al., in preparation). Thus, juvenile fishes used in this study did not produce endogenous steroid hormones. Taken together, the spermatogenesis process observed in this study may have been induced by only exogenous MT.

Spermiation was confirmed in two fishes after 80 days MT treatment together with HCG injection. Besides, sperm motility was observed in the MT-treated fishes that did not release milt. It is necessary to investigate the fertilization ability of sperm in the future. However, it is thought that MT-treated fish is possible to secure the sperm necessary for the artificial insemination of longtooth grouper breeding.

Previous studies demonstrated a dramatic shift in steroidogenesis from endogenous estrogen production to androgen production at the beginning of natural sex change in protogynous hermaphrodites, including groupers, controlled by the endocrine hypothalamus and pituitary signals (Frisch 2004; Kobayashi et al. 2013b; Nagahama et al. 2021). In fact, dynamic sifts of steroid hormone production during natural sex change were involved by follicle stimulating hormone (FSH) produced in the pituitary in honeycomb grouper (Kobayashi et al. 2010). However, whether MT had effects on the pituitary and hypothalamus was not investigated in the present study. Future studies are needed that affect MT on the pituitary and hypothalamus of juvenile grouper to clarify the endocrinological mechanism of artificial sex reversal.

In this study, we used a 2-year-old longtooth grouper, which is easy to handle. Since MT treatments showed a high induction rate for sex reversal, this method is possible in even smaller fish. Indeed, sex reversal has been induced in other grouper species using smaller juvenile fish before gonadal differentiation (Hur et al. 2012; Murata et al. 2014; Wang et al. 2018). However, small fish are considered unsuitable for grouper aquaculture because they are susceptible to handling and may not express good traits.

In our previous study, MT feeding of Malabar grouper larvae (120 days after hatching) can induce sex reversal from immature ovary to testis (Murata et al. 2014). However, the testis reversed to ovary soon after MT treatment withdrawal, as well as orange-spotted grouper (Wang et al. 2018). In contrast, permanent

sex reversal with MT was confirmed in juvenile or adult of other grouper species (Alam et al. 2006; Sarter et al. 2006; Lee et al. 2014). These reports suggest that maintenance of sex-reversed testis may vary with age, gonadal maturation stage, and species difference. Further, we should examine the gonadal sexual stage after MT treatment withdrawal to determine sex-reversed testis will be maintained for an extended period.

Although techniques for breeding longtooth grouper are available (Teruya and Yoseda 2006; Inoue et al. 2014), the problems associated with the quality of seed remain. In order to achieve this, it is necessary to selective breeding such as profitable fast-growth and form. This study established the highly effective method of producing small functional males from the pre-pubertal female by treating MT. Thus, this method may reduce the period of selective breeding. In recent years, harmful consequences on the environmental damage and human health by residues of hormones used in aquaculture had been reported (Hoga et al. 2018). Since only sperm from the MT treated males will be used for grouper breeding, environmental pollution is low in future. However, it is therefore necessary to take care that MT does not leak out of the fish into the environment.

Conclusion

It could be concluded that complete sex reversal from the immature ovary to mature testis can be induced in juvenile longtooth grouper by treatment with MT for 80 days. The proliferation of testicular tissues and degradation of ovarian tissues were observed simultaneously in the sex transitional gonads. Spermiation and sperm motility were confirmed in sex-reversed juvenile longtooth grouper after MT treatment together with HCG injection.

List of abbreviations AI, Aromatase inhibitor; BV, Blood vessels; GSI, Gonad somatic index; GW, Gonad weight; HCG, Human chorionic gonadotropin; OC, Ovarian cavity; PCNA, Proliferating Cell Nuclear Antigen.

Competing interest The authors declare that they have no competing interest.

Author's contribution YK, TM and HC carried out the sampling. YK carried out the histological analysis. HC participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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