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Original Article

Induction of precocious sex reversal in aquaculture: effects of methyltestosterone treatment on gonadal sex of yearling longtooth grouper (*Epinephelus bruneus*)

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Abstract: Groupers, highly valued fish globally, exhibit sex change from female to male during adulthood, posing challenges in obtaining wild males for aquaculture. Inducing female-to-male sex reversal in juvenile groupers can streamline breeding efforts. This study used 17α-Methyltestosterone (MT)-loaded cholesterol pellets to treat one-year-old longtooth groupers (*Epinephelus bruneus*) and induce small functional males. Gonadal sexuality and male functionality were assessed after one to two months. Initial gonadal changes included efferent duct differentiation. High-dose MT-treated fish exhibited active spermatogenesis. However, no spermiation was observed. This highlights MT's potential for sex reversal but not for complete testicular function. These findings have implications for grouper aquaculture and the management of sex change. Further research should explore methods to optimize functional male induction for sustainable breeding practices.

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Introduction

The grouper (Epinephelinae: Serranidae) is traded frequently as a seafood product worldwide because of its highly desirable taste (Rimmer and Glamuzina, 2019). Unfortunately, habitat destruction and increased pressure from overfishing have dramatically reduced wild grouper populations (Sadovy de Mitcheson et al., 2013). Large-scale and sustainable grouper aquaculture has been proposed for biodiversity conservation and the fishery industry. Therefore, grouper aquaculture has been actively pursued in many countries, especially in East Asia (Rimmer and Glamuzina, 2019). In Japan, large-scale aquaculture of several grouper species has been initiated. Currently, 500,000 larvae of longtooth grouper (*Epinephelus bruneus*), a popular species in Japan and Korea, are produced annually in various parts of Japan.

Groupers change their sex from female to male during their life cycle (protogynous hermaphrodite) (Devlin and Nagahama, 2002). This natural sex change is generally observed in individuals of larger sizes and older ages (Bhandari et al., 2003), and hence, retrieval of males from natural resources is limited, and it is difficult to obtain males for aquaculture. Although sex change can be induced artificially in captivity, it requires an extended period. In the case of longtooth groupers, sex change occurs until 15 years of age (10-30 kg body weight) (Chuda et al., 2022). Therefore, for sustainable and efficient grouper aquaculture, it is essential to control the timing of sex change artificially.

In general, changes in balancing endogenous oestrogen and androgen levels are essential for initiating female-to-male sex change in fish (Nakamura et al., 2007; Kobayashi et al., 2013; Soyano et al., 2022). Therefore, artificial sex reversal from female to male in adult groupers has usually been performed using 17a-methyltestosterone (MT) or aromatase inhibitors (Zhou and Gui, 2010). Recently, it has been confirmed in several grouper species that sex reversal can be induced even in immature individuals by MT treatment (Kobayashi, 2022). We have previously reported that induction of sex reversal

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is feasible in juvenile longtooth grouper (2-year-old) (Kobayashi et al., 2021). This technique of induction of precocious sex inversion dramatically helps to manage breeding and to reduce the generation interval. However, the usefulness of precocious sex-reversed males in aquaculture has not been investigated.

The objective of this study was to confirm the usefulness of males from precocious sex reversal as broodstock in aquaculture. For this purpose, it is necessary to confirm successful progression of spermatogenesis and spermiation in sex-reversed males. We first treated yearling longtooth groupers with MT to confirm gonadal changes and spermatogenesis and then investigated the effect of human chorionic gonadotropin (HCG) treatment on spermiation in experimental fish.

Materials and Methods

Ethical approval: This study was approved by the Institutional Animal Care and Use Committee (permission number: A-00399) and carried out accordingly at Kindai University, Nara, Japan. An anaesthetic agent (FA100; Sumitomo Pharma Animal Health Co., Ltd., Osaka, Japan) was used in the experiment to minimise fish suffering.

Experimental fish: The experiment was conducted from May to July 2022. Twelve-month-old longtooth groupers that were raised from artificial seedlings at Kindai University, Shirahama, Japan, were used in this study. The mean body length and weight of the fish used in the experiment were 16.63±0.74 cm and 117.01±15.32 g, respectively. All experimental fish were maintained in a running seawater tank under natural conditions. Pit tags (Biomark, Idaho, USA) were implanted in all fish for individual identification.

Hormonal treatment: Androgen treatment of yearling longtooth groupers was performed using cholesterol pellets. According to a previous report (Lee et al., 1986), androgen-cholesterol pellets were prepared in-house. The synthetic androgen, 17α-methyltestosterone (MT; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in 50% ethanol and mixed with cholesterol powder. The matrix was dried, compounded with molten cocoa butter, and compressed into pellets using a mould. Pellets with two different MT concentrations (MT-low: 2 mg MT/kg BW; MT-high: 10 mg MT/kg BW) were prepared individually according to the body weight of each fish. The control group did not receive any cholesterol pellets. On 12 May 2022, MT-low or MT-high groupers were implanted with pellets containing 2 or 10 mg MT/kg BW, respectively. The MT pellets were implanted into the dorsal muscle of the fish after anaesthesia in 0.2 ml/L FA-100 (Bussan Animal Health Co., Ltd. Osaka, Japan).

Sampling and spermiation: In this study, we conducted sampling before hormone treatment (initial control, N = 5 fish) and one- and two-months following MT treatment (control, N = 9; MT-low, N = 16; MT-high, N = 15 fish). After anaesthesia, the body length and weight of each experimental fish were measured. As the gonads of all experimental fish were small and difficult to remove, the entire abdomen was fixed with Bouin's solution for several hours. The gonads were then trimmed, re-fixed overnight, and stored in 70% ethanol until histological observation.

Spermiation was checked at sampling to confirm male function. Two days before sampling (one- or two-month following MT treatment), human chorionic gonadotropin HCG (Aska Animal Health Co., Ltd. Tokyo, Japan) was injected into all samples to facilitate spermiation. HCG dissolved in a 0.6% NaCl solution was injected into the dorsal muscle (500 IU/kg BW). Before decapitation at the last sampling, spermiation was identified by the milt flowing out of the genital pore when the abdomen was gently massaged.

Gonadal Histology: To analyse the gonadal stage, Bouin's fixed samples from each individual were dehydrated in a series of alcohol, clarified in Fast Solve (FALMA, Tokyo, Japan), and then embedded in paraffin wax. Paraffin blocks were sectioned into 7 μm sections using a rotary microtome (RM2235, Leica Microsystems Inc.) and subjected to haematoxylin and eosin staining according to the usual method. The sections were then observed under a system microscope (FSX100, Olympus Corporation). Based on histological observations, the gonadal sex of each experimental fish

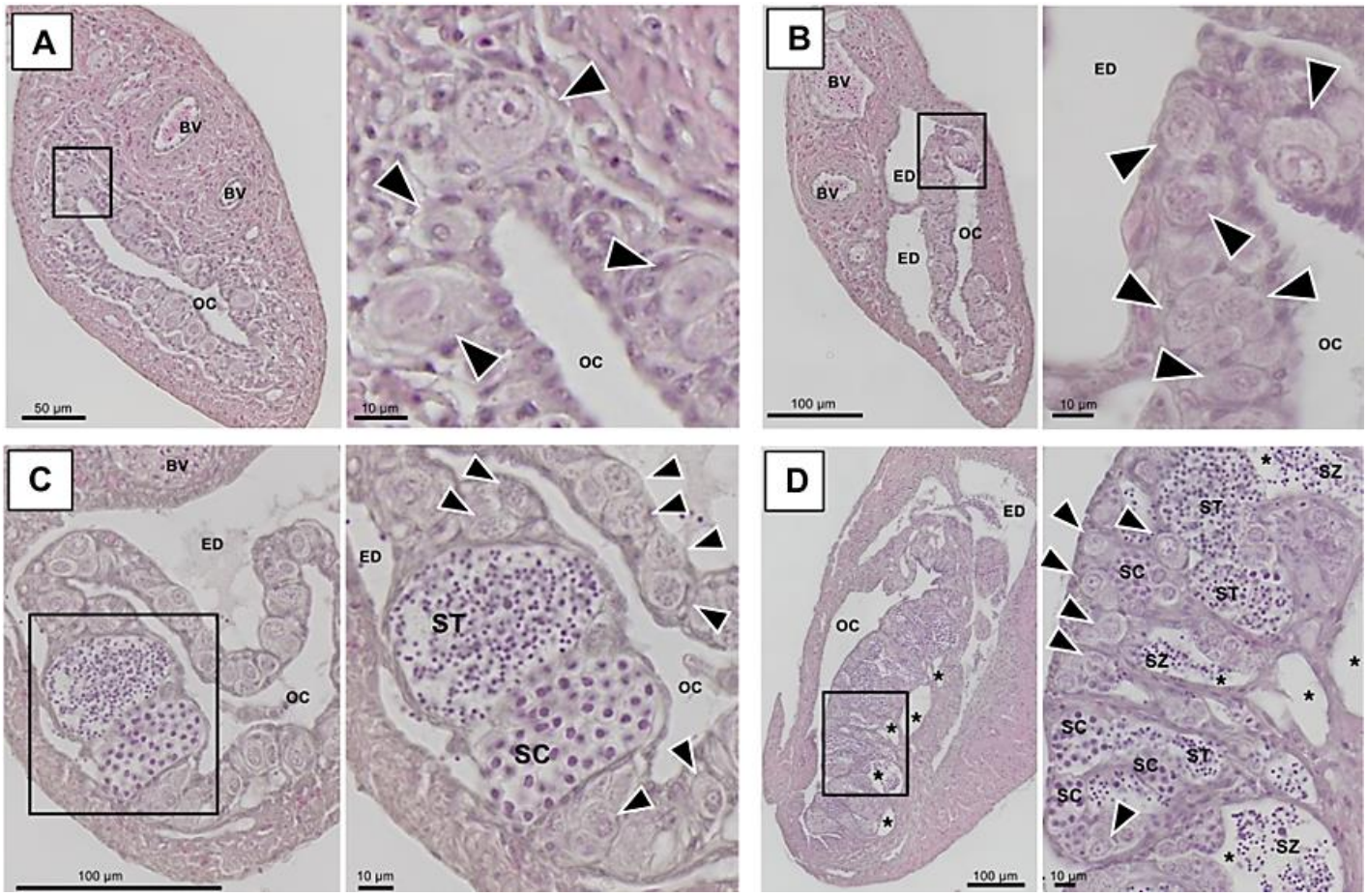

Figure 1. Transverse-sections of gonads of longtooth grouper stained with hematoxylin and eosin. The insets show a higher magnification of the boxed area. A: Immature ovary in female phase, B: gonad in early sex-reversal phase, C: gonad in late sex-reversal phase, D: testis in male phase. The seminiferous tubules are marked with asterisks (*) (Abbreviations: BV, blood vessel; OC, ovarian cavity; ED, efferent duct; SC, spermatocytes; ST, spermatids; SZ, spermatozoa).

was classified into four stages (Fig. 1). The details of gonadal sexuality are described below.

Female phase: Immature ovaries. The central ovarian cavity (OC) is located in gonial germ cells. No vitellogenic oocytes were observed in the gonads (Fig. 1A).

Early sex-reversal phase: The onset of sex reversal is characterised by newly differentiated efferent ducts (ED) in the dorsal region of the gonad. Gonial germ cells and OC were also observed in the gonads (Fig. 1B).

Late sex-reversal phase: In this stage, some gonial germ cells differentiated into spermatocytes (SC) and spermatids (ST) (Fig. 1C).

Male phase: At this stage, increasingly active male germ cells, including spermatozoa (SZ), were observed in the gonads. Simultaneously, seminiferous tubules were also easily recognised in the gonads (Fig. 1D).

Results

Effect of MT implantation on gonadal sex and spermiation of yearling longtooth grouper: To examine the effects of MT on female-to-male sex change in juvenile groupers, we treated yearling longtooth groupers with MT and observed gonadal sex histologically one or two months after treatment (Fig. 2). All fish in the initial and control groups had immature ovaries that contained gonial germ cells. In contrast, induction of sex reversal was observed in all fish treated with MT. In the MT low-dose group, a large proportion of individuals experienced gonadal sex reversal at one- and two-months post-treatment. After two months of treatment, the highest percentage of males was observed in the high-dose MT group.

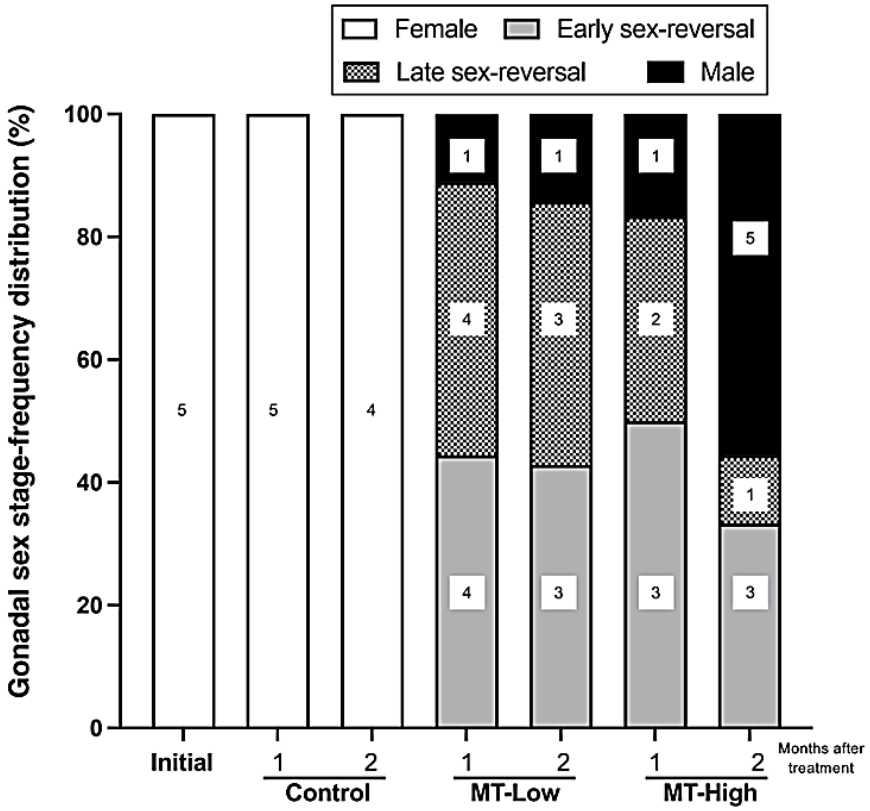

Figure 2. Gonadal sex stage-frequency distribution of longtooth grouper implanted with MT. Numbers in bars indicate the total number of fish.

In an attempt to determine whether precociously sex-reversed individuals can be used as broodstock, all fish were injected with HCG two days before terminal sampling. However, no individuals, including groupers in the male phase, spermiated in this study.

Discussions

Induction of female-to-male sex reversal in juvenile groupers had previously been achieved by exogenous androgen treatment (Kobayashi, 2022). However, the suitability of small sex-reversed males in aquaculture remains unknown. In our study, MT treatment of the longtooth grouper (1-year post-hatching) showed gonadal sex reversal from immature ovaries into complete testes with efferent ducts. There was no evidence of dose dependency of MT on the induction rate of gonadal sex reversal. The induction rate was 100%, which was significantly higher than that reported in other groupers (Yeh et al., 2003; Kobayashi, 2022), suggesting that MT treatment with cholesterol pellets was effective. However, no spermiation was observed, indicating that the MT-treated fish were non-functional males. Similarly, MT treatment prior to sexual differentiation has been reported to induce long-term infertility in other gonochoristic species (Paul-Prasanth et al., 2013; Sun et al., 2014; Weber et al., 2020). The cause of infertility in the sex-reversed juvenile longtooth grouper is possibly due to insufficient growth of their gonads. Current research suggests that endogenous oestrogen is involved in testicular growth in teleosts (Schulz et al., 2010). Low doses of oestrogen stimulate testicular growth in black porgy and wrasse (Wu et al., 2008; Kobayashi et al., 2011). In addition, the simultaneous administration of oestrogens and androgens has been found to induce sexual reversal in groupers (Huang et al., 2019). Therefore, simultaneous estrogen/androgen treatment of juvenile groupers may produce small functional males.

In general, gonadal sex change in protogynous hermaphrodites first occurs in the regression of ovarian tissue, followed by the proliferation of testicular tissue (Nakamura et al., 2005; Kobayashi et al., 2013). In this study, gonial germ cells and somatic cells in the ovaries of juvenile longtooth groupers directly differentiated

into testicular tissues during sex reversal. This result indicates that gonial germ cells and somatic cells in juvenile ovaries have high sexual plasticity, similar to what has been observed in other teleost fish (Yoshizaki et al., 2010; Liu et al., 2017).

Vitellogenesis in fish is regulated by endogenous oestrogen (Lubzens et al., 2010). In this study, no vitellogenic oocytes were observed in the initial or control groups. Thus, endogenous oestrogen production was thought to be almost non-existent in fish in this study. Therefore, it is suggested that the direct differentiation of gonial germ cells in the ovaries into sperm is induced only by exogenous androgen treatment.

It has been suggested that the differentiation of the efferent ducts and the proliferation of testicular tissue coincide during sex change in adult groupers (Nakamura et al., 2005; Alam and Nakamura, 2007;). In the present study, however, because the gonads of the juvenile fish were small and the entire gonad could be observed, it became clear that the first morphological change during sex reversal was the differentiation of the efferent ducts. This is in accordance with the fact that in mammals, the efferent ducts and initial segment of the epididymis are highly responsive to androgens (Oliveira et al., 2004; Joseph et al., 2011). To date, studies on sex-changing fish have focused on the fate of germ cells and their somatic cells in the gonads (Nakamura et al., 2007), and the details of the changes to the genital ducts are largely unknown. It is well known that genital ducts (ovarian cavity, oviduct, efferent duct, and sperm duct) are essential for the function of the gonads (gamete final maturation and release). Therefore, it is necessary in the future to elucidate the mechanisms responsible for the differentiation of the genital ducts during the process of sex change.

Conclusion

The induction of gonadal sex reversal was confirmed in all MT-treated yearling longtooth groupers. However, all fish were non-functional males, in that milt was not released when the abdomen was gently massaged. These results suggest that further development of technology for inducing small males by MT treatment is needed for application in grouper aquaculture.

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