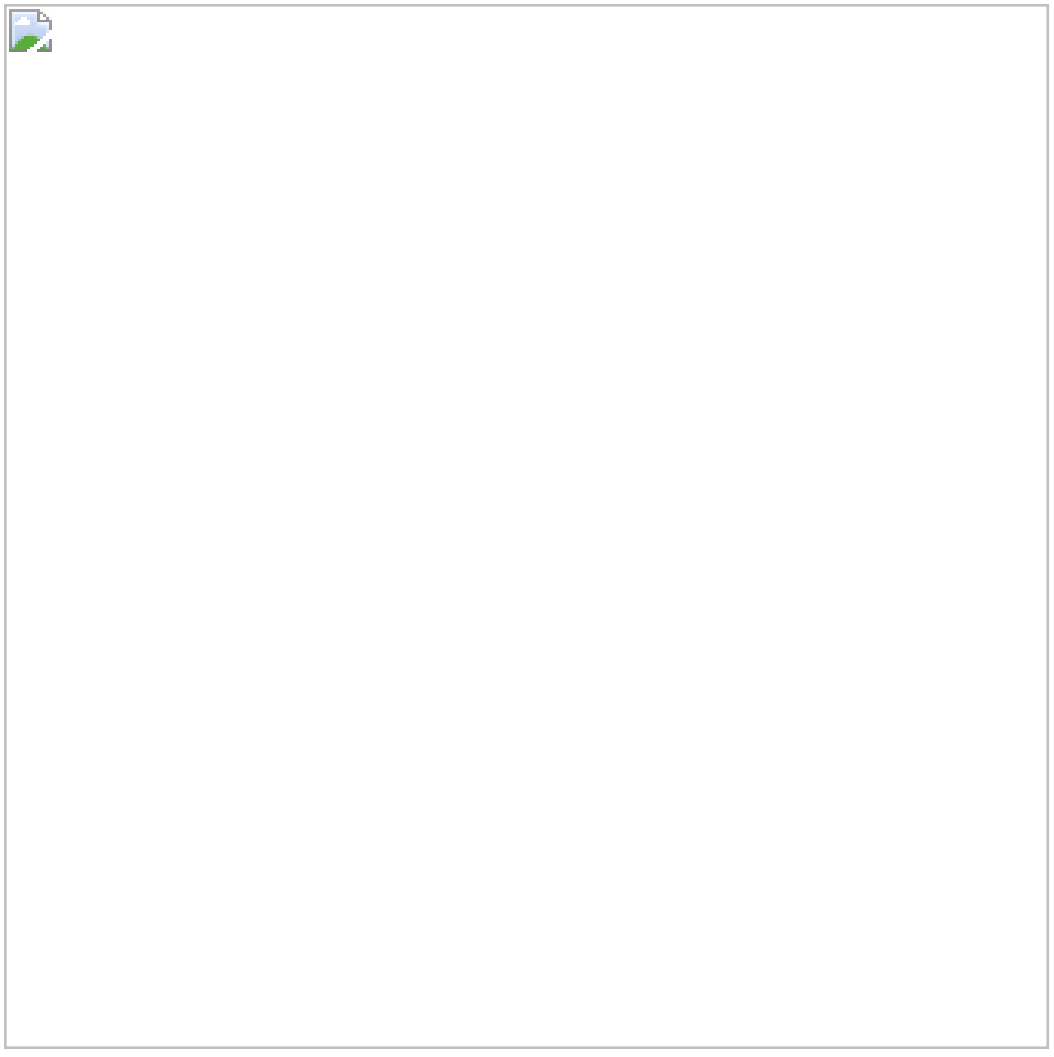

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Sex Inversion of Sexually Immature Honeycomb Grouper (*Epinephelus merra*) by Aromatase Inhibitor

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ABSTRACT—Previous studies have shown that estrogen plays an important role in sex change of protogynous honeycomb grouper, and that the treatments with aromatase inhibitor (AI) cause estrogen depletion and complete sex inversion of pre-spawning females into functional males. In the present study, we examined whether AI causes sex inversion of sexually immature females. Female honeycomb groupers were implanted with various doses of Fadrozole (0, 100, 500 and 1000 μg/fish) in the non-breeding season, and resultant changes in the gonadal structures and the plasma levels of sex steroid hormones (estradiol-17β, E2; testosterone, T; 11-ketotestosterone, 11-KT) were examined three months after implantation. Vehicle-implanted groups did not change sex, while 100 and 500 μg AI-implanted groups had turned into transitionals with intersex gonad. In contrast, the highest dose receiving group exhibited both transitional and male phases. Transitional phase gonad had atretic oocytes and spermatogenic germ cells at the late stages of spermatogenesis, while male phase testis contained spermatozoa accumulated in the seminiferous tubules. All males released sperm upon slight pressure on the abdomen. In the AI-implanted fish, plasma levels of E2 decreased in a dose-dependent manner, while the levels of 11-KT were high in the highest dose receiving group. Present results suggest that estrogen plays an important role in sex change of protogynous honeycomb grouper, and that treatments with AI potentially inhibits endogenous E2 production *in vivo*, causing oocyte degeneration and subsequently the sex inversion from female to male. The Fadrozole could be an important tool for manipulating the sex of hermaphrodite fishes.

Key words: sex change, hermaphrodite fish, honeycomb grouper, aromatase inhibitor, estrogen

INTRODUCTION

Teleost fishes exhibit a wide variety of reproductive tactics, including adult sex change. Gonochoristic fishes, after gonadal differentiation at the early stages of life cycle, do not reverse sex, while hermaphrodite fishes change their sex in response to social stimuli, age, growth rate and seasons. The honeycomb grouper (*Epinephelus merra*) is a protogynous hermaphrodite fish. Due to its relatively smaller size among groupers, this species serves as a good model for grouper research. The sex change occurs at an adult stage, particularly in the non-breeding season. In the field populations of honeycomb groupers, the onset of sex change was associated with the oocyte degeneration and low levels of serum estradiol-17β, which was subsequently accompanied by gradual increase in the 11-KT levels and spermatogonia proliferation toward the center of ovigerous lamellae (Bhandari *et al*., 2003). In the protogynous wrasse (*Thalassoma duperrey*), the onset of sex change was associated with oocyte degeneration and significant drop in E2 levels (Nakamura *et al*., 1989). These results suggest that the endogenous level of estrogens, when drop below the threshold levels *in vivo*, could be stimulatory to sex change in the field populations of protogynous hermaphrodite fishes.

In fish, estrogen selectively acts as an ovarian inducer (Nakamura *et al*., in press). The estrogen biosynthesis is mediated by steroidogenic enzyme cytochrome P450 aromatase (arom), which is believed to play an important role in ovarian differentiation of various fish species (Nakamura *et al*., 1998, 2000; Guigen *et al*., 1999; Kitano *et al*., 2000; D'Cotta *et al*., 2001). The aromatase inhibitor (AI) treatments around the period of gonadal sex differentiation have caused estrogen depletion and various degrees of masculinization, including complete and functional sex reversal in some species (Piferrer *et al*., 1997; Kitano *et al*., 2000; Kwon *et al*., 2000, 2002; Afonso *et al*., 2001). In hermaphrodite fishes, AI treatments of adult females have resulted in either partial (Kroon and Liley, 2000) or complete sex reversal (Higa *et al*., in press; Bhandari *et al*., in press). However,

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very little is known about effects of AI on sex inversion of sexually immature hermaphrodite fishes. In the present study, we collected female honeycomb groupers in the non-breeding season and implanted them with various doses of non-steroidal AI (Fadrozole) to elucidate the role of estrogen in sex change of protogynous hermaphrodite fishes.

MATERIALS AND METHODS

Wild honeycomb groupers of average total length of 18 to 20 cm were collected from fishermen of Nakijin village, northern Okinawa. We have found that female honeycomb groupers have total length below 20 cm (Bhandari *et al.*, 2003). The spawning season starts during the full moon of May and lasts in July (Soyano K, personal communication). Generally, October through January are considered as non-breeding period. In the present study, sexually immature females were selected to implant with AI (a gift from Norvartis Pharma, Tokyo, Japan) and as controls. Altogether, 42 fish were implanted, of which 10 fish were sacrificed for initial control, and remaining 32 fish were divided into four groups with eight fish in each treatment. On January 6, 2003, control fish received implantation of vehicle (cocoa butter only) and fish in the treatment groups received 100, 500 and 1000 μg AI (dissolved in the cocoa butter), respectively into the body cavity. Later, they were reared in the polyethylene cages (80 cm×80 cm×80 cm) placed over 5 tons cemented tanks with flow-through sea water. All fish were sampled in April 6, 2003. After anaesthetization with phenoxyethanol (0.05%), the total length and wet body weight were measured, blood was collected from caudal vein into a 1 ml heparinized syringe, and fish were sacrificed for the collection of pituitary and gonads. Gonads were weighed to determine gonadosomatic index (GSI) and a small piece of gonad from each individual was preserved in the Bouin's solution for the histological analysis. The collected blood was centrifuged at 3000 rpm for 15 minutes and obtained plasma was stored at –30°C until analysis. The plasma levels of E2, testosterone (T) and 11-ketotestosterone (11-KT) were determined by enzyme-linked immunosorbent assay (ELISA) according to the routine procedure. Later, preserved gonadal tissues were embedded in the paraffin, sectioned at 7 μm, and stained with Delafield's hematoxylin and eosin. The prepared slides were examined under light microscope for histological changes.

RESULTS

GSI and Histological changes in gonads

At the end of experiment, the GSI of control fish was significantly higher than that in the remaining groups (Fig. 1A). The gonadal restructuring in the AI-implanted fish were categorized into four stages: female, early transition, late transition and male, according to Bhandari *et al* (2003). At the start of experiment, the ovary of initial control females had oocytes at the perinucleolar stages, except some at the pre-vitellogenic stages (Fig. 2A). At the end of experiment, the ovary of control females had vitellogenic oocytes at the yolk globule stage (Fig. 2B). A fish from control group had ovotestis at the early transition stage. About 75% of 100 μg AI-implanted fish had gonads at early transition stage, where perinucleolar stage oocytes were undergoing atresia, and spermatogonia were sparsely proliferating at the periphery of the ovigerous lamella, indicating sex change (Fig. 2C). In the intersexual gonads of 500 μg AI-implanted fish,

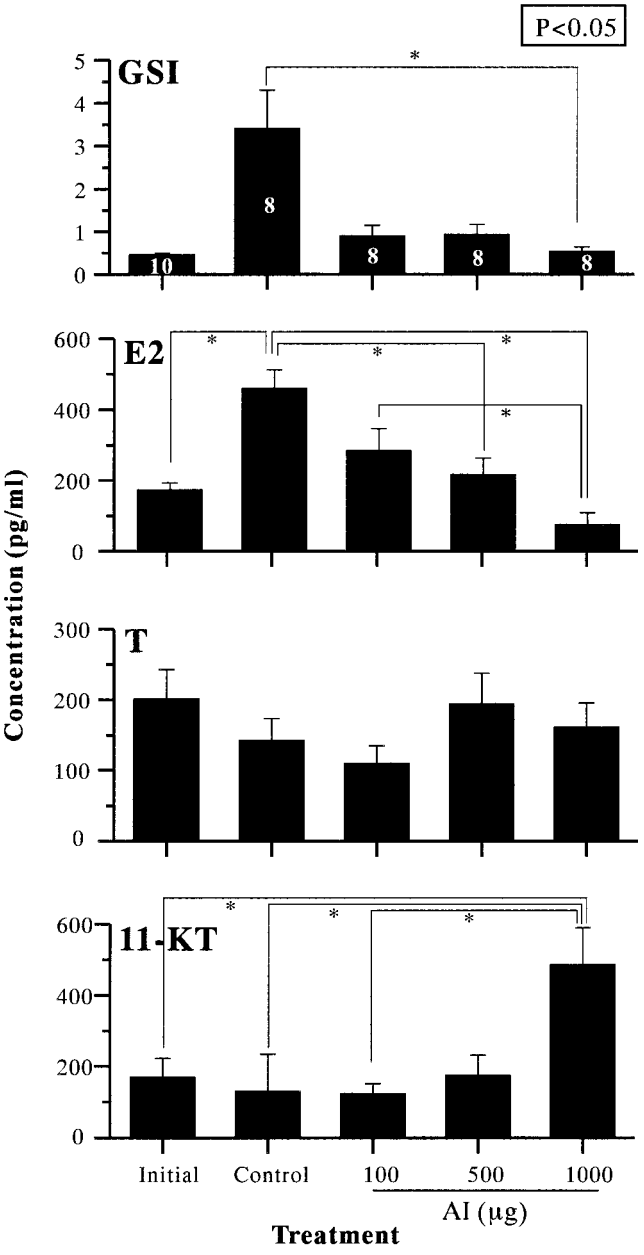

Fig. 1. Changes in gonadosomatic index (GSI), plasma levels of estradiol-17β (E2), testosterone (T) and 11-ketotestosterone (11-KT) in the honeycomb grouper implanted with various doses of Fadrozole. Numbers in the bars indicate total number of fish sampled. * indicates statistically significant difference at $P<0.05$ from the connected means.

spermatogenic germ cells were extensively proliferating, and previtellogenic oocytes were undergoing atresia, indicating the sex change was progressing (Fig. 2D). Out of eight 1000 μg AI-implanted fish, the gonads of two fish were at the early transition stage, three were at the late transition stage, and three had already transformed into testis. Those testes had various stages of spermatogenic germ cells, including an accumulation of sperm in the seminiferous tubules (Fig. 2E). The degree of sex change in each treatment is shown in the Fig. 3. The gonads of majority of 100

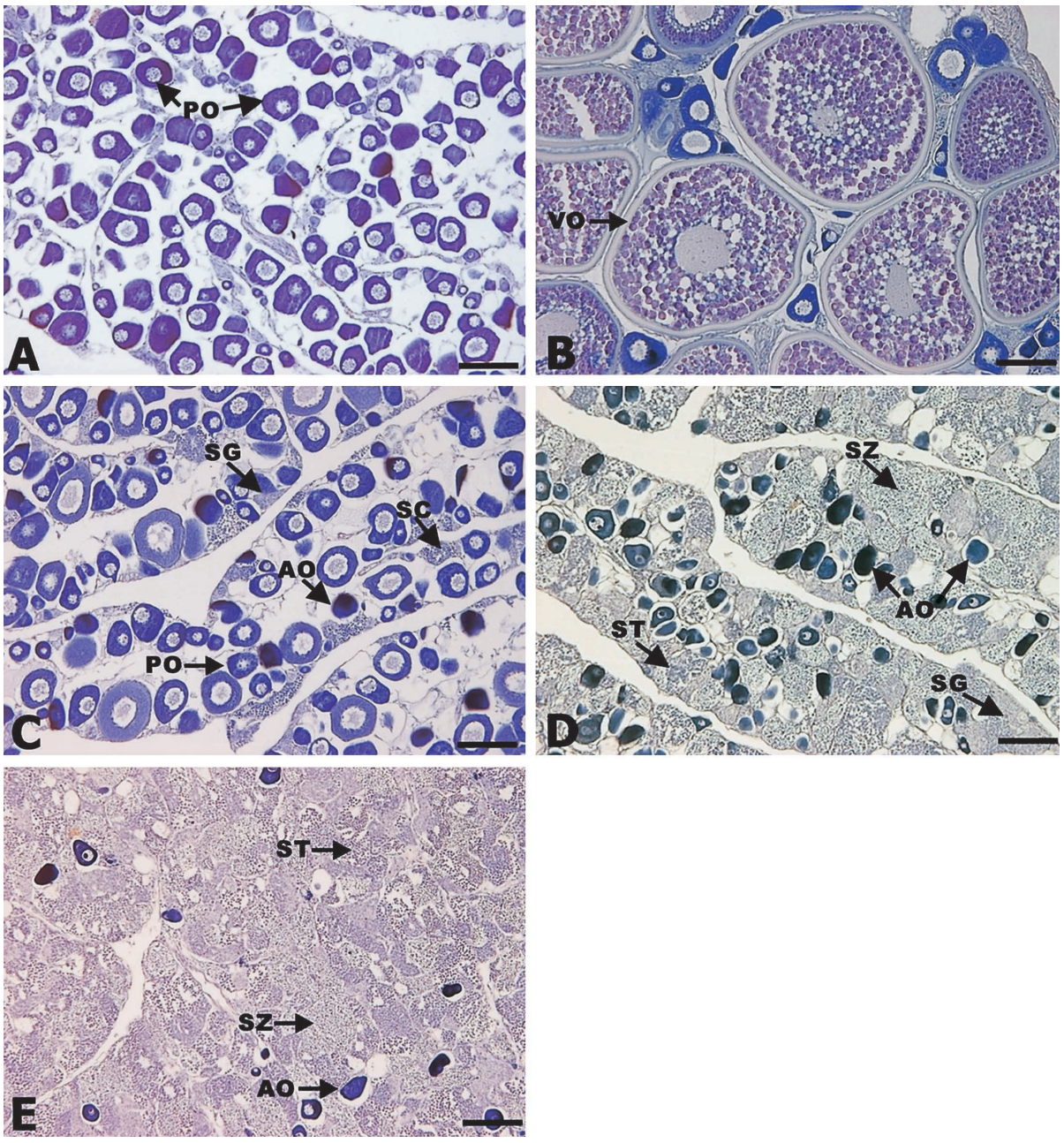

Fig. 2. Light micrograph of gonadal structure of honeycomb grouper before and after implantation with Fadrozole: (A) initial control (B) vehicle, (C) 100 μg, (D) 500 μg, and (E) 1000 μg Fadrozole/fish. Abbreviations: AO, atretic oocyte; PO, primary oocyte; VO, vitellogenic oocyte; SG, spermatogonia; SC, spermatocyte; ST, spermatid; and SZ, spermatozoa. Scale bar=100 μm.

and 500 μg AI-implanted fish were at early or late transition stages, whereas those in 1000 μg AI-implanted fish were at the late transition stage or fully transformed into testis.

Changes in the plasma levels of sex steroid hormones

The plasma levels of sex steroid hormones are shown in the Fig. 1. Compared to initial controls, the plasma levels of E2 significantly increased in the control fish (Fig. 1), whereas in the AI-implanted fish, the levels decreased in a dose-dependent manner. The plasma levels of T did not change, while the levels of 11-KT significantly increased in the 1000 μg AI-implanted fish.

DISCUSSION

In the present study, we investigated whether AI causes sex inversion of sexually immature female honeycomb grouper. The low doses of AI-receiving fish had shown the progress in sex change, whereas the majority of highest dose receiving fish had either late transitional gonad or transformed into testis, suggesting AI has dose-dependent effects on sex inversion of honeycomb grouper.

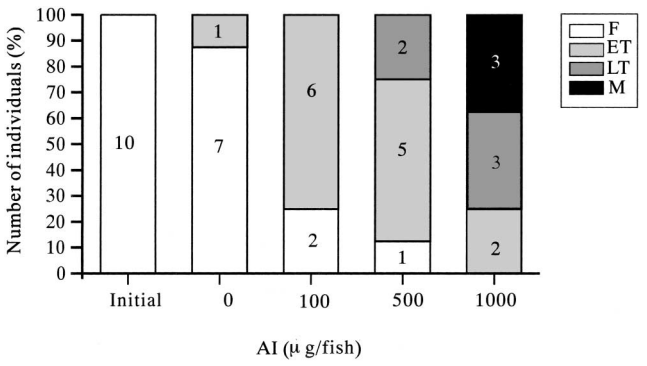

Fig. 3. Sex ratio of honeycomb grouper implanted with various doses of AI. Abbreviations: F, Female phase; ET, early transition phase; LT, late transition phase; M, Male phase (according to Bhandari *et al*., 2003). Numbers in bars indicate the total number of fish. The testes of LT and Male phase fish contained spermatogenic germ cells at various stages of development, including spermatozoa.

The steroidogenic enzyme aromatase that converts androgens into estrogens plays an important role in ovarian differentiation (Nakamura *et al*., 1998; Strüssmann and Nakamura, 2002; Guigen *et al*., 1999, D'Cotta *et al*., 2001). The AI inhibition of aromatase activity is believed to cause oocyte degeneration, and induce sex reversal in various fish species. The efficacy of AI has been tested in a variety of gonochoristic species, where treatments around the period of gonadal sex differentiation have caused complete sex reversal from females to functional males (Piferrer *et al*., 1997; Nakamura *et al*., 1999; Kitano *et al*., 2000, Afonso *et al*., 2001), while its effects on sex reversal of adult gonochorists are largely unknown. In contrast, in the hermaphrodite fishes, AI treatments have shown variable results in terms of its effectiveness. For the honeycomb grouper, AI-implantation caused complete sex inversion of pre-spawning females to functional males in two and half months (Bhandari *et al*., in press), while the oral administration caused complete sex inversion of wrasse in six weeks (Higa *et al*., unpublished), whereas the treatments for the same period caused formation of transitional gonads in the blackeye goby (Kroon and Liley, 2000). Together with previous results, present results support the hypothesis that AI treatments cause sex inversion of protogynous hermaphrodite fishes; however, the effectiveness probably depends on species, the extent of gonadal development, and the dose of AI.

In the wild honeycomb grouper, low levels of serum E2 and the degeneration of perinucleolar stage oocytes were associated with the onset of sex change (Bhandari *et al*., 2003), while in the wrasse, *Thalassoma duperrey,* a rapid drop in E2 levels, and degeneration of pre-vitellogenic oocytes were observed at the onset of sex change (Nakamura *et al*., 1989). In the present study, the plasma E2 levels were decreased dose- dependently in the AI-implanted fish. Treatments with AI caused a significant drop in the E2 production by ovarian fragments of sexually mature female honeycomb grouper *in vitro*, while AI-implantation of sexually maturing females caused E2 depletion and led to complete and functional sex inversion *in vivo* (Bhandari *et al*., in press), whereas E2 compensation preserved the female sex (Bhandari *et al*., unpublished). Together, present results clearly suggest that a significant drop in endogenous estrogen levels is necessary to trigger the female to male sex change in fish.

In fish, estrogen is believed to act as ovarian inducer. In the gonochoristic Nile tilapia, AI treatments, after the period of gonadal sex differentiation, caused sex reversal of genetically controlled all females into functional males (Nakamura *et al*., in press), whereas E2 prevented AI-induced sex reversal (Bhandari *et al*., unpublished). Other than fish, AI inhibition of estrogen synthesis has caused sex reversal of variety of animals, such as Newt (Chardard and Dournon, 1999), reptiles (Dorizzi *et al*., 1994; Wibbles and Crews, 1994), and chicken (Elbrecht and Smith, 1992; Abinawanto *et al*., 1996; Burke and Henry, 1999). Moreover, the role of estrogen on female sex differentiation in mammals has been explained by creating estrogen receptor knock-out mice. The ovaries of estrogen receptor α and β knock-out mice exhibited follicle transdifferentiation to structures resembling seminiferous tubules of the testis, including Sertoli-like cells and expression of mullerian inhibiting substance, sulfated glycoprotein-2, and Sox9 (Couse *et al*., 1999). These results strongly support the present results that estrogen is inevitable for maintaining female sex in the wide range of vertebrate classes, where depending on the stages of sexual development, any alteration of estrogen balance could result in sex reversal.

In the present study, plasma 11-KT levels were increased in the sex-changed males, while no change was observed for T levels. Although, the levels of 11β-hydroxytestosterone (11β-HT) could not be measured in plasma, it is likely that testosterone might have converted into 11β-HT as T to 11β-HT conversion is active during sex change in some hermaphrodite fishes (Idler *et al*., 1976). In the wild population of honeycomb grouper, 11-KT levels tended to increase with the developmental stages of testicular tissues (Bhandari *et al*., 2003), while in the wrasse the 11-KT levels increased sharply after the onset of sex change (Nakamura *et al*., 1989). In the field populations of red grouper, 11-KT levels were high in the individuals possessing inter-sex and male gonads (Johnson *et al*., 1998). In addition, treatments with 11-KT or methyltestosterone or the mixture of various androgens have resulted in complete sex change of several protogynous hermaphrodite fishes (Tan-Fermin *et al*., 1996; Kroon and Liley, 2000, Tanaka *et al*., 2000; Yeh *et al*., 2003a, b; Higa *et al*., in press). These results clearly indicate that androgens also play important role in the sex change of hermaphrodite fishes, but yet, there still remain some questions: whether increase in 11-KT levels exert stimulatory effects on spermatogonia proliferation in the ovary or stimulate spermatogenesis after its complete transformation into testicular tissues. Nevertheless, future development of protocol for *in vitro* sex change would be helpful for elucidating

the cellular mechanism of germ cell proliferation during sex reversal and sex change in fish.

In conclusion, present results support the hypothesis that E2 plays an important role in maintaining female sex of hermaphrodite fishes, and that the AI inhibition of E2 synthesis causes oocyte degeneration and subsequently the spermatogonia proliferation in the ovary leading to functional sex inversion. Moreover, Fadrozole being a potent aromatase inhibitor (Steele *et al*., 1987; Smith, 1999), could be an important tool for the sex inversion of hermaphrodite fishes and also in the studies to further investigate what determines the fate of a gonocyte to develop into either oogonia or spermatogonia during the onset of sex change or reversal. In the aquaculture point of view, the AI-induction of sex change would be an important tool for the timely production of male brood stocks for breeding purposes and to enhance grouper aquaculture.

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