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Short term treatment with aromatase inhibitor induces sex change in the protogynous wrasse, *Halichoeres trimaculatus*

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ABSTRACT

The purpose of this study was to specify the time when individuals are committed to female to male sex change in the protogynous wrasse, *Halichoeres trimaculatus*, induced by treatment with the nonsteroidal aromatase inhibitor (AI) Fadrozole. In this study, treatment with AI was carried out by providing adult females with a diet containing 500 μg AI/g food for 3 (AI-3), 5 (AI-5), and 10 days (AI-10). We examined the gonadal structure of the fishes histologically at the end of the AI treatment and 30 days after the start of the experiment. At the end of the AI treatment, all individuals in the AI-3 treated group had gonads with degeneration of yolky oocytes, indicating the onset of sex change. Most individuals in the AI-5 treated group had gonads with atretic vitellogenic oocytes, like those in AI-3 treated group, whereas most individuals in the AI-10 treated group had gonads with testicular tissue. At 30 days after the onset of the experiment, approximately 70% of the individuals in the AI-3 treated group had mature ovaries, whereas all fishes in AI-5 and AI-10 treated groups had mature testes, indicating sex change. Therefore, treatment with AI for only 5 days resulted in complete sex change. Our results also indicate that crucial events for testicular differentiation occur within 5 days from the start of AI treatment. Thus, we conclude that females are committed to change into males after 5 days of AI treatment.

1. Introduction

In addition to gonochorism, several types of hermaphroditism are common in fish (Atz, 1964; Yamamoto, 1969; Ross, 1990; Devlin and Nagahama, 2002). Sequential hermaphroditism, an individual changing sex during its life cycle, is especially common. Sequential hermaphroditism is subdivided into female-to-male (protogyny), male-to-female (protandry), and serial sex change. Although many protogynous species are known, the physiological mechanisms underlying sex change are not well understood.

Recent studies indicate that steroid hormones play important role in protogynous sex change. In the saddleback wrasse, *Thalassoma duperrey* (Labridae), plasma estradiol-17β (E2) levels dropped rapidly at the onset of sex change (Nakamura et al., 1989). In the protogynous Mediterranean red porgy, *Pagrus pagrus* (Sparidae), plasma E2 levels at the early transition phase were significantly lower than in breeding females (Kokokiris et al., 2006). These facts indicate that a decrease in endogenous estrogens plays a critical role in the initiation of sex change. Estrogen depletion in females by treatment with aromatase inhibitor (AI) also brings about sex change to male. AI treatment has been carried out in several gonochoristic and hermaphroditic fishes. Also, in some gonochoristic fishes, AI induced testicular differentiation of undifferentiated gonads in genetic females (Kitano et al., 2000; Kwon et al., 2000; Afonso et al., 2001; Komatsu et al., 2006). In protogynous fish, adult females treated with AI started sex change (Bhandari et al., 2004a,b; Alam et al., 2006; Benton and Berlinsky, 2006), and in the protandrous black porgy, AI blocked sex change from male to female (Lee et al., 2002). These results strongly suggest that estrogens play important roles in sex change.

The three-spot wrasse, *Halichoeres trimaculatus* (Labridae), which inhabits coral reefs in Okinawa, is a protogynous fish. This species exhibits diandry, and populations consist of small initial-phase (IP) males, IP females, and large terminal-phase (TP) males. TP males have territories and pair-spawn with females. Sex change in this species is controlled by the social system. When a TP male disappears from a territory, the largest females within the territory change sex from female to male. In aquarium experiments, the largest females changed sex within 38 days of introduction of four females into each tank (Kuwamura et al., 2007). It is also reported that the social system controls sex change in some fishes (Fishelson, 1970; Ross et al., 1990; Warner and Swearer, 1991; Mackie, 2003; Walker and McCormick, 2004). However, the physiological mechanisms of sex change are not known. In a previous study of the three-spot wrasse, females administered AI orally for 6 weeks changed sex completely to males (Higa et al., 2003). This result

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indicates that a decrease in estrogens plays a critical role in sex change in this species. However, the initial step of sex change was not focused on in that study, so when sex change is triggered remains unclear. Additionally, Higa et al. (2003) did not follow the process of the sex change histologically. The purpose of the present study is to identify the time when females are committed to change into males during treatment with AI. In addition, we examined the gonads of these fishes during sex change to document the process histologically.

2. Materials and methods

2.1. Animals

Fishes were collected by roll net on the coast of Motobu, northern Okinawa, Japan, at the beginning of July, 2006, and in the middle of June, 2007. The fishes were transferred to and maintained at Sesoko Station. To sex the fishes, the abdominal region of fish was pressed, and fishes that did not release sperm were assumed to be females. Females were distributed into 500-L polyethylene tanks (ranging from 28.5 to 30.2 °C in 2006, and from 28.0 to 31.4 °C in 2007). The fishes, seven or eight females per tank, were acclimatized for 2 weeks with flow through sea water under natural photoperiod and were fed an artificial diet (C-2000; Kyowa Hakko Kogyo, Ltd., Tokyo, Japan). To block social system-induced sex change, one TP male was placed in each tank.

2.2. AI treatment

Fadrozole (nonsteroidal AI; Novartis Pharma Inc., Tokyo, Japan) was dissolved in ethanol and mixed with the artificial diet at the dose of 500 μg/g. Fishes in the AI treatment groups were fed this diet twice daily during the experiments.

2.3. Experimental design

Before AI treatment, eight fishes were sacrificed as an initial control group. AI was administered for 3 days (AI-3), 5 days (AI-5), and 10 days (AI-10). Each of the treatment groups had a corresponding control group. AI-3 treatment was carried out at the beginning of July, 2007, and AI-5 and AI-10 treatments were performed at the end of July, 2006. At the start of AI treatment, each AI treatment group had two tanks where seven or eight females were placed. Fishes in one tank were sampled at the end of AI treatment. To check whether each AI treatment induced irreversible sex change, fishes in the other tank were autopsied after 30 days from the onset of the experiment. There was some mortality in the AI-3 (one fish) and AI-10 (two fishes) treated groups during the experiment. After the experiment, histological analysis revealed that three IP males were present in the experimental groups (AI-10 control group at the end of AI treatment, AI-3 and AI-10 treated groups after 30 days from the onset), so the data on these three fishes were excluded.

2.4. Sampling procedures

After anesthetization with 0.01% 2-phenoxyethanol, the total length, the standard length, and body weight of each fish were measured. Blood was collected from the caudal vein using a 1-ml heparinized syringe (Terumo, Japan) and was centrifuged at 12,000 rpm for 10 min to obtain plasma. Plasma was stored at −30 °C until analysis. Gonads were removed and weighed to determine the gonadosomatic index (GSI: gonad weight/body weight * 100). The gonads were fixed overnight in Bouin's solution at room temperature, then dehydrated in a series of alcohols, cleared in benzene, and embedded in paraffin. Cross-sections (7 μm) were stained with hematoxylin and eosin following conventional histological procedures and were examined by light microscopy.

2.5. Classification of gonadal stages in sex change

Nakamura et al. (1989) classified the process of sex change in *T. duperrey* into six stages based on histological observations. The six stages of sex change are: stage 1, a normal ovary with vitellogenic oocytes and previtellogenic oocytes, but no testicular tissue; stage 2, an ovary with degenerating vitellogenic oocytes, but normal young oocytes; stage 3, disappearance of vitellogenic oocytes, degeneration of previtellogenic oocytes, and development of somatic cells in the central region of the lamellae; stage 4, gonad with proliferation of presumed spermatogonia and remains of a few degenerate oocytes; stage 5, gonad with increased spermatogenesis and disappearance of atretic oocytes at the peri-nucleolus stage; stage 6, mature testis with active spermatogenesis and spermiation proceeding in all parts of the testis with an ovarian cavity. In the present study, the process of sex change in *H. trimaculatus* induced by AI treatment corresponded to the process of sex change in *T. duperrey*. We therefore applied to the classification of sex change in *T. duperrey* to the present results.

2.6. Steroid assay

Serum 17β-estradiol (E2) was determined by enzyme-linked immunosorbent assay (ELISA) as described by Asahina et al. (1995).

2.7. Statistical analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for statistical analysis of GSI and plasma E2 levels.

3. Results

3.1. Histological observations

All individuals in the initial control had normal ovaries containing vitellogenic oocytes and previtellogenic oocytes, and no apparent testicular tissue (Fig. 1A). Histological observations at the end of AI treatment are summarized in Table 1. At the end of AI treatment, six out of seven individuals in the AI-3 control had mature ovaries. The remaining individual had an immature ovary without vitellogenic oocytes. All individuals in the AI-3 treated group had gonads with degenerating yolky oocytes indicating the onset of sex change, but no testicular tissue (Fig. 1B). All individuals in the AI-5 control had mature ovaries with vitellogenic oocytes and previtellogenic oocytes. In the AI-5 treated group, four out of seven individuals had ovaries with degenerating oocytes at stage 2 (Fig. 1C). Another had a gonad containing many degenerate oocytes at the peri-nucleolus stage and few degenerate yolky oocytes (stage 3; Fig. 1D). The remnants of degenerate oocytes, clusters of somatic cells, and a few gonial germ cells were observed in the lamellae in this gonad. Another individual had a gonad with a few degenerating oocytes and some presumed spermatogonia on the outer periphery of the ovigerous lamellae (stage 4; Fig. 1E). The other had a testis with active spermatogenic germ cells and with an ovarian cavity, indicating sex change (stage 6). All gonads in the AI-10 control were mature ovaries. Two out of eight fishes in AI-10 treated group had gonads at stage 3. These gonads had degenerating young oocytes and a few gonial germ cells. Three fishes had gonads at stage 4, characterized by proliferation of presumed spermatogonia and