Artificial Sex Reversal of White Grouper (*Epinephelus aeneus*) Utilising Aromatase Inhibitor (Fadrozole)

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Running Head: Artificial Sex Reversal in White Grouper

ABSTRACT

The white grouper is a desirable aquaculture species that adapts to captivity, grows well and commands a high market price. However, little is known about reproductive biology or control of sex reversal of this protogynous hermaphrodite. In this study female white groupers were implanted with one dose 17α-methyltestosterone (10 mg/kg body weight (BW), MT) and two doses of aromatase inhibitor, fadrozole (1 and 3 mg/kg BW, FD1 and FD3) once a month for four months (April-July). At the start of the study, the fish had gonads full of oocytes compared to the end of the experiment when the control group mature oocytes compared to the experimental groups MT, FD1 and FD3 that exhibited different stages of testicular tissue. Plasma levels of testosterone were significantly highest in the FD3 group and the highest 11-Ketotestosterone levels were observed in the MT group. Plasma levels of estradiol (E_2) were significantly lower in the fadrozole implanted groups, compared to initial individuals and control groups. The use of aromatase inhibitor, fadrozole for sex reversal both gives further insight into the mechanisms controlling sex differentiation and provides an alternative to steroid treatment.

KEYWORDS

Aromatase inhibitor, sex reversal, white grouper, steroids

1. INTRODUCTION

Many *Epinephelus* species are threatened and have been included in the International Union for Conservation of Nature (IUCN) red list of threatened species. The white grouper (*Epinephelus aeneus*) in the Mediterranean Sea region is not an exception and are threatened by an increasing consumer demand. In Southeast Asia grouper culture has been implemented and is taking advantage of the species of grouper that have rapid growth, disease resistant and efficient feed conversion (Ranjan *et al*., 2013). Decreasing amounts of wild fish and bans on the fishing of groupers in the Mediterranean region prompted studies targeting culture of native grouper species, such as the white grouper. An important aspect to developing grouper culture is an

understanding of reproductive biology to control the sex reversal of these protogynous hermaphrodite species. Normally, the sex-change of groupers depends on their size/age or their complex social behaviour (Munday, Buston & Warner, 2006). Due to paucity in the supply of 6-10 kg mature male white groupers in the wild and the cost of waiting for spontaneous masculinization in captivity, studies of captive broodstocks have been focused on artificial sex reversal (Zhou & Gui, 2010).

In protogynous hermaphrodites, as in other teleosts, sex reversal is regulated by endocrine signalling through hypothalamus-pituitary-gonad (HPG) pathway (Kobayashi, Murata & Nakamura, 2013). The gender of the fish is determined and differentiated by the particular sex steroid produced in the gonads, as a target organ, in response to the hypothalamus-pituitary endocrine transmission (Kobayashi *et al*., 2013). Studies administering exogenous synthetic androgens to females of mainly Asian grouper species (*E. tauvina, E. bruneus, E. malabaricus, E. akaara* and *E. coioides*) induced a sex reversal from female to male (Yeh, Kuo, Ting & Chang, 2003; Sarter, Papadaki, Zanuy & Mylonas, 2006; Murata, Karimata, Alam & Nakamura, 2010; Hur *et al*., 2012). Testosterone (T), 11-Ketotestosterone (11KT) and synthetic 17α-methyltestosterone (MT) have been used for induce a female to male sex reversal and MT can be considered the most effective and commonly used female to male sex reversal steroid in teleost fishes (Robert & Schlieder, 1983; Chao & Chow, 1990; Tan-Fermin, 1992; Mylonas & Zohar, 2001). Glamuzina, Glavic, Skaramuca & Kozul, (1998) obtained testicular tissue in various phases of spermatogenesis from dusky grouper (*E. marginatus*) after feeding with 5 mg/kg BW MT during 3 months. Genç, Aktaş, Eroldoğan & Genç (2016) demonstrated that dusky grouper (*E. marginatus*) were induced to change sex to permanent males using a 60 days treatment of 11.5 mg MT/kg BW implanted at 30 days intervals. Red-spotted grouper implanted with 10 mg/kg BW MT had gonads in early transitional stages after 4 weeks (Li, Liu & Lin, 2006a). Peatpisut & Bart (2010) demonstrated that orange-spotted grouper (*E. coioides*) injected with 4 mg/kg BW MT converted into functional males within 120 days. Our previous study showed that after 10 weeks goldblotch grouper (*E. costae*) implanted with 5 mg/kg BW of MT each month changed to phenotypic males and fish implanted with 10 mg/kg BW of MT each month had seminiferous tubules (Yılmaz *et al*., 2015).

Further studies have revealed the efficacy of non-steroidal agents for the masculinization of females. The cytochrome P450 aromatase is an enzyme that converts C19 androgens; testosterone and androstenedione, into C18 estrogen, estradiol (E_2) and estrone, respectively (Seralin & Moselemi, 2001; Diotel *et al*., 2010; Tsai, Lee, Chen & Chang, 2011; Murata *et al*.,

2011). Fadrozole (FD), an aromatase inhibitor, induced complete sex change in honeycomb grouper (*E. merra*), through inhibition of estrogen biosynthesis and perhaps the subsequent induction of androgen function (Bhandari, Komuro, Higa & Nakamura, 2004a). Garcia *et al*. (2013) demonstrated that using aromatase inhibitor was effective to obtain functional male dusky grouper (*E. marginatus*), during the breeding season. Furthermore, lower serum estradiol and higher testosterone levels were obtained after FD implantation in red-spotted grouper (*E. akaara*) when compared with the control group (Li, Liu, Zhang, & Lin, 2006b). In addition, successful sex reversal was achieved in juvenile longtooth groupers (*E. bruneus*) at week 7 post FD injection, and gonads initiated spermatogenesis (Hur *et al*. 2012). According to these findings, FD may inhibit estradiol production and induce a change in sex from female to male in other grouper species.

To our knowledge, no study has been undertaken in the induction of sex reversal of white grouper with fadrozole administration. In this study, we aimed to assess, for the first time, the efficacy of long-term treatment with 17α-methyltestosterone and fadrozole on sex reversal of white grouper.

2. MATERIALS AND METHODS

All fish handling procedures complied with Turkish guidelines for animal care (No. 28141) set by the Ministry of Food, Agriculture and Livestock.

2.1. Experimental fish and design

A total of 25 juvenile wild white groupers obtained from İskenderun Bay were transported to Marine Research Station of Faculty of Fisheries of Cukurova University (Adana, Turkey) and used in this study. Fish were quarantined, fed and acclimatised for 4 months before the study began. Fish were fed with frozen sardine 6 days a week during acclimatization and individually marked with a passive integrated transponder (PIT) tag (AVID, Uckfield, East Sussex, UK) for identification. At the onset of the study the body weight (BW) of the fish ranged between 326-725 g; and the length (L) between 31-39.5 cm. Throughout the study, fish were maintained in

an indoor tank (15 m^3) with a recirculating system and exposed to a natural photoperiod. Ammonium, nitrite and nitrate levels were kept under critical levels (NH_3<0.1 mg/L, NO_2<0.1, NO_3<0.5). The salinity, pH and oxygen levels of the water varied between 36-38 ‰, 7.0-8.5 and 7.5-8.0 ppm respectively. Daily recirculating water exchanged was 400 % of tank volume. The water temperature in the tank was kept close to the natural seawater temperature from 21 to 28ºC (Figure 1). Fish were fed with frozen sardine or squid 4 days a week and with moisture diet 2 days a week comprising sardine, krill and rice powder, vitamin C, vitamin and mineral mix and guar gum as a binder. The ingredients of moisture feed were modified from Sugama *et al*. (2012) method used for grouper broodstock. The feeding frequency was once a day at a feeding rate of 2 % of body weight.

At the beginning of the experiment, three fish (~356 g) were sampled as an initial group. Fish were allocated into four groups; group FD1 (n=6) and FD3 (n=6) were those implanted with 1 and 3 mg/kg BW doses of Fadrozole (Fadrozole hydrocloride, Sigma-Aldrich), respectively, group MT (n=6) were implanted with 10 mg/kg BW dose of 17α-Methyltestosterone (Sigma-Aldrich, St. Louis, MO, USA) and the control group (n=4) was implanted with a placebo implant. All groups were kept in the same tank throughout the study. The study period was 4 months.

2.2. Preparation and application of MT and FD implants

FD and MT were mixed with ethanol and subsequently with cholesterol, (Sigma-Aldrich, St. Louis, MO, USA) and left over-night at room temperature. The next day, FD and MT were blended with cacao oil in a ratio of 1:5 (cacao oil:hormone cholesterol mix). During blending the mixture was kept at room temperature to avoid the cacao oil melting. Once mixed completely, each hormone mixture was poured into a mould and pressed to form a 2.5 mm diameter pellet. For placebo implants, for the control group, the same procedure was undertaken but without addition of MT or FD. The implants were stored at 4 ºC till use. Prepared as slices cut from the cylindric pellets, the hormone content of each implant was adjusted according to the mass of the individual fish. After fish were anesthesized with 200 mg/L of 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), weighed total length of fish was measured at each implantation. Thereafter, prepared pellets were implanted subcutaneously in the lateral dorsal muscle region of the fish utilising an implantation syringe. Taking into

consideration the annual reproductive season of the white grouper, which starts in July and ends at the end of August in the Mediterranean (Bouain & Siau, 1983), implantations were started at the beginning of April and continued monthly until the beginning of August (Figure 1).

2.3. Samplings

Groups were sampled for circulating hormones, gonadosomatic index and histological examination of gonads at the beginning of the study in April (02.04.15) and at the end of the study in August (03.08.15). Samplings were performed following anaesthetise with 200 mg/L of 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA). The length (L) and body weight (BW) of each fish were measured. Blood samples were taken from the caudal vein by using sterile, heparinized (Nevparin, 25.000 IU/5ml) syringes. Plasma were separated by cooled centrifugation (Hettich R220) at 3000 rpm for 10-15 min at +4ºC and stored at -20ºC until analysis for sex steroids. Gonads were dissected out and massed for calculation of the Gonadosomatic Index (GSI)=gonadal weight/(total body weight-gonadal mass) x 100). Dissected gonads were fixed and stored in 10% formaldehyde buffer solution until histological processing and examination.

2.4. Histological examination and sex steroid assays

Gonads fixed in formaldehyde solution were transferred sequentially into 70, 85, 95 % ethanol baths aliquots. Dehydrated gonads were made transparent by using xylene before embedding into paraffin. Following minimum 4 hours at +4º C, the blocks were sectioned transversely into widths of 4-7 µm, by using microtome (Thermo Shandon, Germany). Sections were then stained with hematoxylin-eosin (H&E), sealed with Entellan (Merck, Darmstadt, Germany) and observed under light microscope (Leica).

The E_2 and T plasma levels were measured by chemiluminescence method and 11-KT by enzyme-linked immunosorbent assay (ELISA). The T and E_2 Kits were purchased from Berkman Coulter Company (USA) and 11-KT from Cayman Chemical Company (USA). The hormone assays were performed following the manufacturer's instructions.

2.5. Statistical analysis

The data of growth parameters, gonadosomatic index (GSI) and sex steroids were presented as means ± one standard error of the mean (SEM) and analysed with one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test (Gündoğdu, 2014). Differences were regarded as significant when $p < 0.05$. Homogenity of variance was tested with Levene's variance homogenity test. Covariance analysis (ANCOVA) was used for the comparison of the slope of regression curves both mass and length increment. A linear equation was used to describe the relationship between weight/length increment and time. All statistical analyses were carried out using the SPSS V20 (SPSS, Chicago, IL, USA).

3. RESULTS

3.1. Growth, gonadosomatic index (GSI) and histological changes in gonads

During the experiment, there was no mortality among the experimental groups. In the present study, there was a significant effect of the treatment on both length and body weight of fish (Figure 2). As a result of ANCOVA, the slope of time dependent mass ($F_{(4, 95)}=34.6$, $p < 0.05$) and length increment was found to be significantly different ($F_{(4, 95)}=25.5$, $p < 0.05$). Furthermore, determination constant (R^2) of time and increment of body weight/total length between groups were high (Figure 2). Overall, average body weight of fish in FD1 and FD3 and control were significantly higher than MT groups (Figure 2) ($F_{(3, 10)}= 5.451$, $p < 0.05$). Final total length of the FD1 and FD3 groups was higher than other groups ($F_{(3, 10)}= 5.357$, $p < 0.05$). Consistent with this, the highest wet weight gain was observed in the FD3 group (617.0±29.9 g), while the lowest performance was found in MT group (171.3±8.7 g) ($F_{(3, 10)}= 29.901$, $p < 0.05$).

There were no significant differences in GSI among experimental groups except FD3 group. The GSI calculated for the FD3 group was significantly higher than the initial group, control, MT, and FD1 groups (Figure 3) ($F_{(4, 5)}= 54.709$, $p < 0.05$).

The treatments affected the sexual differentiation of gonadal development and the stages of gametogenesis observed (Figure 4). At the start of the experiment all fish had ovarian development with primary oocytes within the lamellas that project towards the lumen (Figure 4a) and were completely female with no testicular tissue. The control group fish sampled at the end of the experiment had oocytes in different stages of development (primary to mature oocyte stages) (Figure 4b). Control group fish similar to the fish at the start of the experiment were completely female at the end of the experimental period. At the end of the experiment, the gonad of MT treated group exhibited testicular tissue (Figure 4c). The 1 mg/kg BW FD implanted fish (FD1) were in transitional phase characterized by reversal from ovarian to testicular tissue. In addition, FD1 fish had a few degenerated oocytes and different stages of spermatogenic germ cells (Figure 4d). The gonads of FD3 group fish, were fully differentiated into testes and were absent of ovarian elements. The testes were undergoing an active spermatogenesis including the presence of spermatids and spermatozoa. The FD3 fish that had running milt, had accumulation of sperm in the seminiferous tubules (Figure 4e).

3.2. Changes in the plasma levels of sex steroid hormones

Mean plasma levels of T were significantly higher in FD3 groups (1.5 ng/mL) than all other groups, followed by the levels in the MT (0.5 ng/mL) and FD1 (0.3 ng/mL) groups ($F_{(4, 12)}$= 28.435, $p < 0.05$) (Figure 5). However, the highest 11KT levels were observed in the MT groups (26.7 pg/mL), while the lowest values were found in the initial (2.5 pg/mL) and control groups (2.6 pg/mL) ($F_{(4, 13)}$= 9.576, $p < 0.05$). Mean plasma levels of E_2 were significantly lower in the fadrozole implanted FD1 (1.5 pg/mL) and FD3 (1.4 pg/mL) groups, compared to the initial (20.4 pg/mL) and control groups (16.3 pg/mL) ($F_{(4, 15)}$= 7.009, $p < 0.05$). However, MT group (4.7 pg/mL) showed lower levels of E_2 than only initial group ($p < 0.05$).

4. DISCUSSION

The present study provides the first evidence that fadrozole successfully induce sex reversal in white grouper. Specifically, by utilising FD implants at a dose of 3 mg/kg BW sex reversal to mature males was induced in white grouper. Generally, previous studies have indicated that, in

successive trials, exogenous sex steroid administrations, particularly MT, were utilized to successfully induce sex reversal in various grouper species (Sarter *et al*., 2006; Murata *et al*., 2010; Genç *et al*. 2016), consistent with the findings in the MT group in the present study. However, also in agreement with the present study aromatase inhibitors were shown to provide an alternative to steroid hormones for inducing sex reversal. In *E. tauvina*, Ranjan *et al*. (2013) demonstrated combination of MT and letrozole, an aromatase inhibitor, to be more effective than using MT alone. Li *et al*. (2006a) revealed that the gonads of more than half of the fish implanted with 10 mg/kg BW MT were in early transitional stages of sex inversion, whereas gonads of more than half of fish implanted with MT+FD were in late transitional stages. Li *et al*. (2006a) explained this outcome by endogenous aromatization of MT to estrogen. However, determining the interactive effect of aromatase inhibitors and androgens behind sex reversal is a complex subject due to the intricate relationships among species-specific, nutrition, and temperature. Thus, it clearly warrants further future investigations for white grouper broodstock management.

In the present study, the fish in the FD3 group had the higher GSI compared to the other groups. Moreover, there were no difference among the initial, control, MT and FD1 groups, in terms of GSI gain. It is known that GSI values are significantly correlated with maturation of gonads and ambient temperature (Li, Liu & Lin, 2007) and photoperiod. In this study, water temperature was maintained close to that of the sea water where the species naturally matures and the same natural photoperiod was preserved. The GSI was similar in the initial and control groups. This may be explained by the fish all remaining in an immature stage (~ 664 g) or that the sampling date was late (in the beginning of August) in the reproductive period and all fish had passed an advanced stage of maturation. Li *et al*. (2006a) demonstrated that GSI's of the MT, 17α-methyldihydrotestosterone and FD+MT implanted red-spotted groupers were significantly lower than those of the initial and control groups after four weeks. Similarly, fadrozole implanted honeycomb groupers showed lower GSI than that of the control group (Bhandari, Komuro, Higa & Nakamura, 2004b). Contrarily, Yeh *et al*. (2003) observed that low doses of androgen (1 and 10 µg/kg BW) stimulated an increase in overall gonadal mass compared to the control and high dose androgen (100, 1000, 10000 and 20000 µg/kg BW) treated groups. Taken together all these studies including the present study, have demonstrated that GSI appears low in intersex stage, which is characterised by absorbed oocytes and signs of onset of spermatogenesis. Normally, the functional testes are smaller than ovaries in orange-

spotted groupers (Yeh *et al*., 2003). However, in our study, FD3 group had significantly higher GSI that are also a characteristic of mature male gonads.

In the present study, evidence of primary oocytes in the initial group, oocytes developments in the control group and testicular tissue in MT group were observed in the gonads. These gonadal developmental stages were consistent with the observations of GSI. In FD1 group, fish were in intersex-sex stage characterised by various stages of spermatogenesis. The FD3 group fish did not have ovarian tissue and the gonads had developed into testes with spermatids and spermatozoa. Similar stages of development were observed in gonads of honeycomb groupers (Bhandari *et al*., 2004a,b; Alam & Nakamura, 2007), longtooth groupers (Hur *et al*., 2012) and dusky groupers (Marino, Azzuro & Massari, 2001; Garcia *et al*., 2013) induced by FD and MT.

The balance between estrogen and androgen levels plays a major role in sex change by inducing steroidogenesis (Zhou & Gui, 2010; Kobayashi *et al*., 2013). Nakamura, Kobayashi, Miura, Alam & Bhandari, (2005) demonstrated high serum levels of E_2 in females in the breeding season and low E_2 levels outside of the breeding season and during the transition stage to males. In the honeycomb grouper, female to male sex change was shown to be associated with a decrease in E_2 levels followed by an increase in androgen levels (Bhandari, Alam, Soyano & Nakamura, 2006). In our study, even though MT group had lower plasma levels of E_2 than the initial group, Fadrozole implanted groups showed lowest E_2 levels compared to both the initial and control groups. The decrease in E_2 levels and simultaneous increase in T levels can be attributed to the intrinsic mechanism of aromatase inhibitor, which inhibits the conversion of T to E_2. This outcome is consistence with Bhandari's findings (2006) and also explains the levels of T in our study, which were highest in the FD3 group. Contrary to expectations, we found that highest 11KT levels in MT treated group. The steroid, 11KT is known to be a fish-specific androgen with the main function of stimulating spermatogenesis (Bhandari, Komuro, H., Higa, Nakamura & Nakamura, 2003; Miura & Miura, 2003). However, in androgen treated orange-spotted grouper plasma 11KT concentrations did not change significantly in fish with various sex stages (Yeh *et al*., 2003). Further research on the physiological function of 11KT is needed.

Apart from inducing sex reversal to mature males in white grouper, to our knowledge, this is the first study investigating the efficacy of exogenous hormone administration on growth rate of white grouper. Statistically significant differences in growth were found between groups over the course of experiment. Time-dependent variance curve of our data revealed that Fadrozole implanted group achieved a faster growth rate than that of MT treated group. Viñas,

Asensio, Cañavate & Piferrer, (2013) also found that the growth and total length of Senegalese sole (*Solea senegalensis*), treated with the synthetic non-aromatizable androgen 17α-methyldihydrotestosterone and fadrozole, significantly increased 196 days' post fertilization and onwards. Contrary to above mentioned study and our present growth/length data, body weight and total length of orange-spotted grouper (*E. coioides*) induced orally and with implantation of MT and fadrozole did not have significant affects on growth (Wu, Tey, Li & Chang 2015).

These findings are expected to provide useful information for improving broodstock management and to induce sex reversal of white grouper. In the light of this result, female white grouper can be sex reversed to males that produce sperm that could be used to establish egg, larvae and juvenile production for aquaculture. Further studies are needed in order to establish white grouper in the aquaculture sector as an alternative species. In conclusion, the observation of semen during the dissection of the gonads, appearance of spermatozoa in histological sections of gonads, lower E_2 and higher T levels in plasma of 3 mg/kg fadrozole implanted fish revealed that this agent at this particular dose is effective in inducing a change from female to male gender.

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464

FIGURE LEGENDS

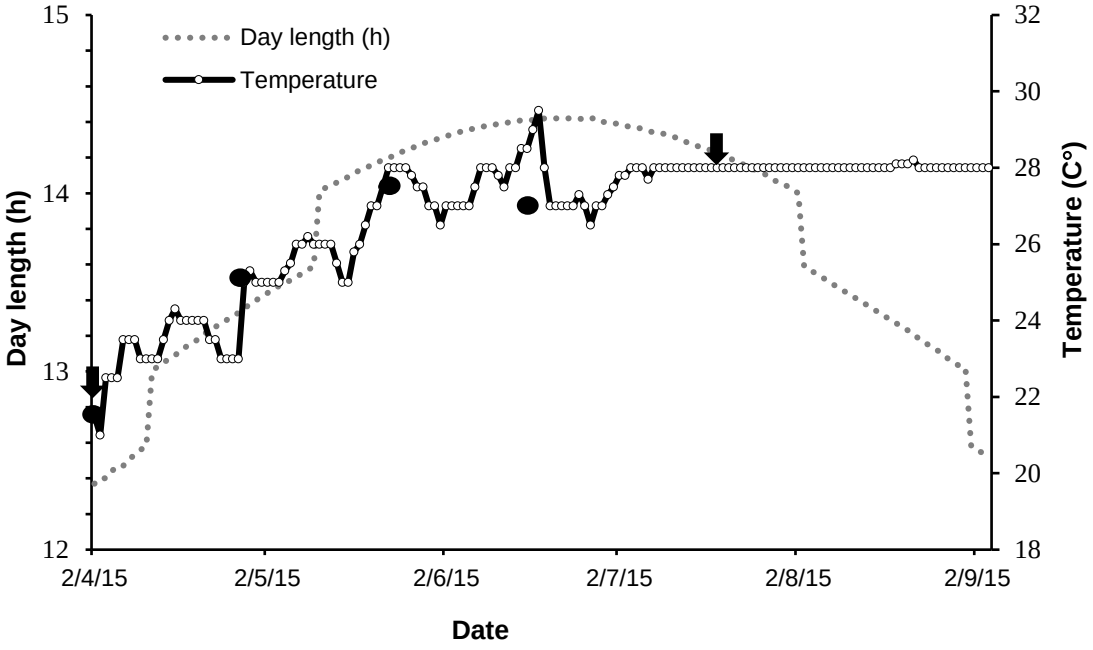
Figure 1. Water temperature and day length profile for the white grouper (*Epinephelus aeneus)* broodstock monitored in the present study. Filled circles and arrows show that implantation and sampling date, respectively.

Figure 2. Growth of white grouper (*Epinephelus aeneus)* over time and as a function of treatment. Linear equations of body weight (a) / length (b) increments depending on time in different groups of white grouper. Slopes are significantly different (ANCOVA, $p<0.05$). At each time, uppercases indicates significant differences between treatment groups (one-way ANOVA, $p<0.05$). Abbreviations: MT, 17α-Methyltestosterone (10 mg/kg BW); FD1-FD3, (1 and 3 mg/kg BW implanted) Fadrozole.

Figure 3. Gonadosomatic index (GSI) of white grouper (*Epinephelus aeneus*) after aromatase inhibitor (1 and 3 mg/kg BW Fadrozole; FD1, FD3) and 17α-Methyltestosterone (10 mg/kg BW; MT) implantation. Values with different letters indicate significant differences ($P<0.05$).

Figure 4. Gonadal histology of white grouper (*Epinephelus aeneus*) (**a)** initial samples (April); **(b):** control group (August); **(c):** 17α-Methyltestosterone (10 mg/kg BW; MT) implanted group **(d):** aromatase inhibitor (1 mg/kg BW Fadrozole; FD1) implanted group **(e):** aromatase inhibitor (3 mg/kg BW Fadrozole; FD3) implanted group. ***O*:** oocyte, ***L*:** lumen; ***PO*:** primary oocyte, ***OD***: Oocyte development, ***SD*:** sperm development in seminiferous tubules filled with spermatid, ***ST*:** seminiferous tubules (H&E, X4, Bar: 500 µm).

Figure 5. Plasma 11-ketotestosterone (11KT), testosterone (T) and estradiol (E2) levels in controls, aromatase inhibitor (1 and 3 mg/kg BW Fadrozole; FD1, FD3) and 17α-Methyltestosterone (10 mg/kg BW; MT) implanted Epinephelus aeneus. Values with different letters indicate significant differences ($P<0.05$).


Day length (h)
Temperature
Day length (h)
Temperature (C°)
Date
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14
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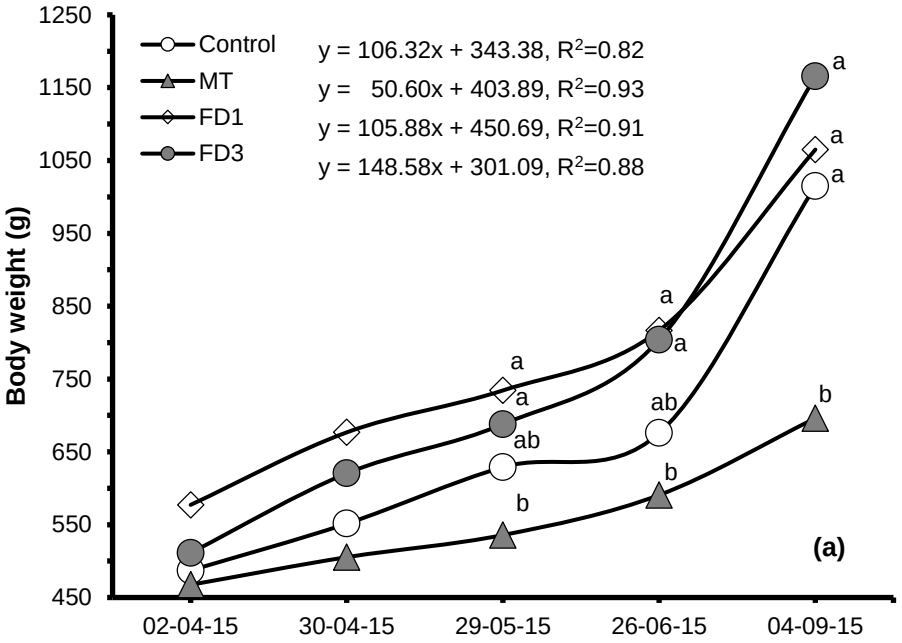
2/9/15

Figure 1.



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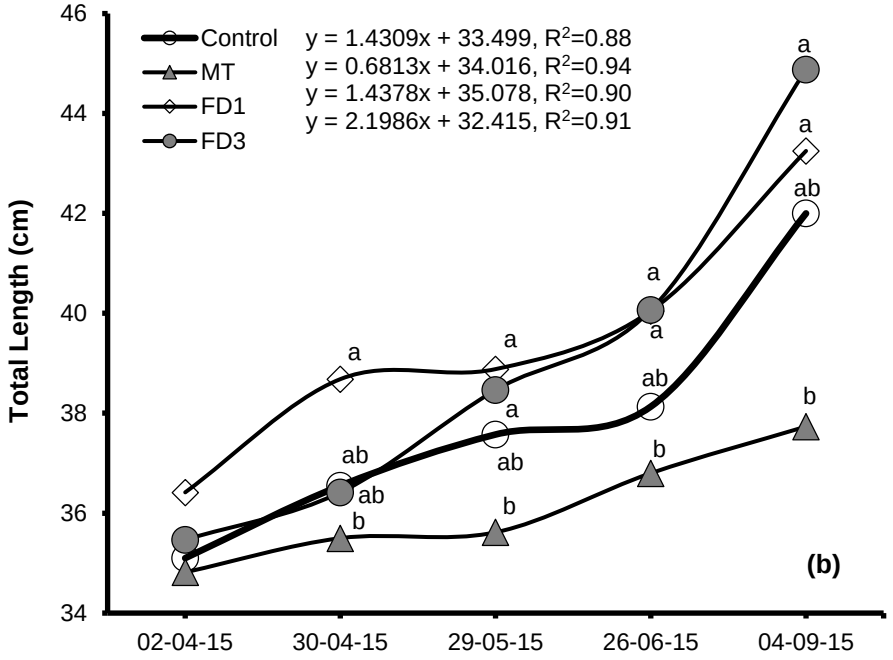

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510 **Figure 2.**

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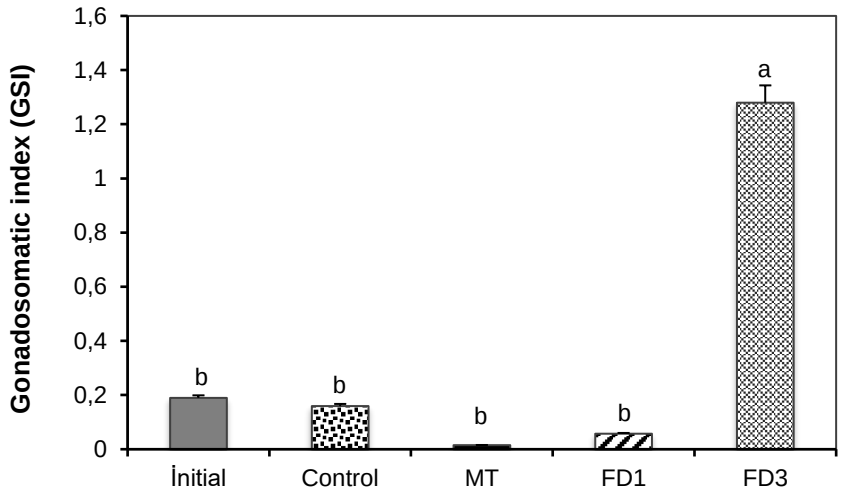
Gonadosomatic index (GSI)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
b
b
b
b
a
İnitial
Control
MT
FD1
FD3

Figure 3.

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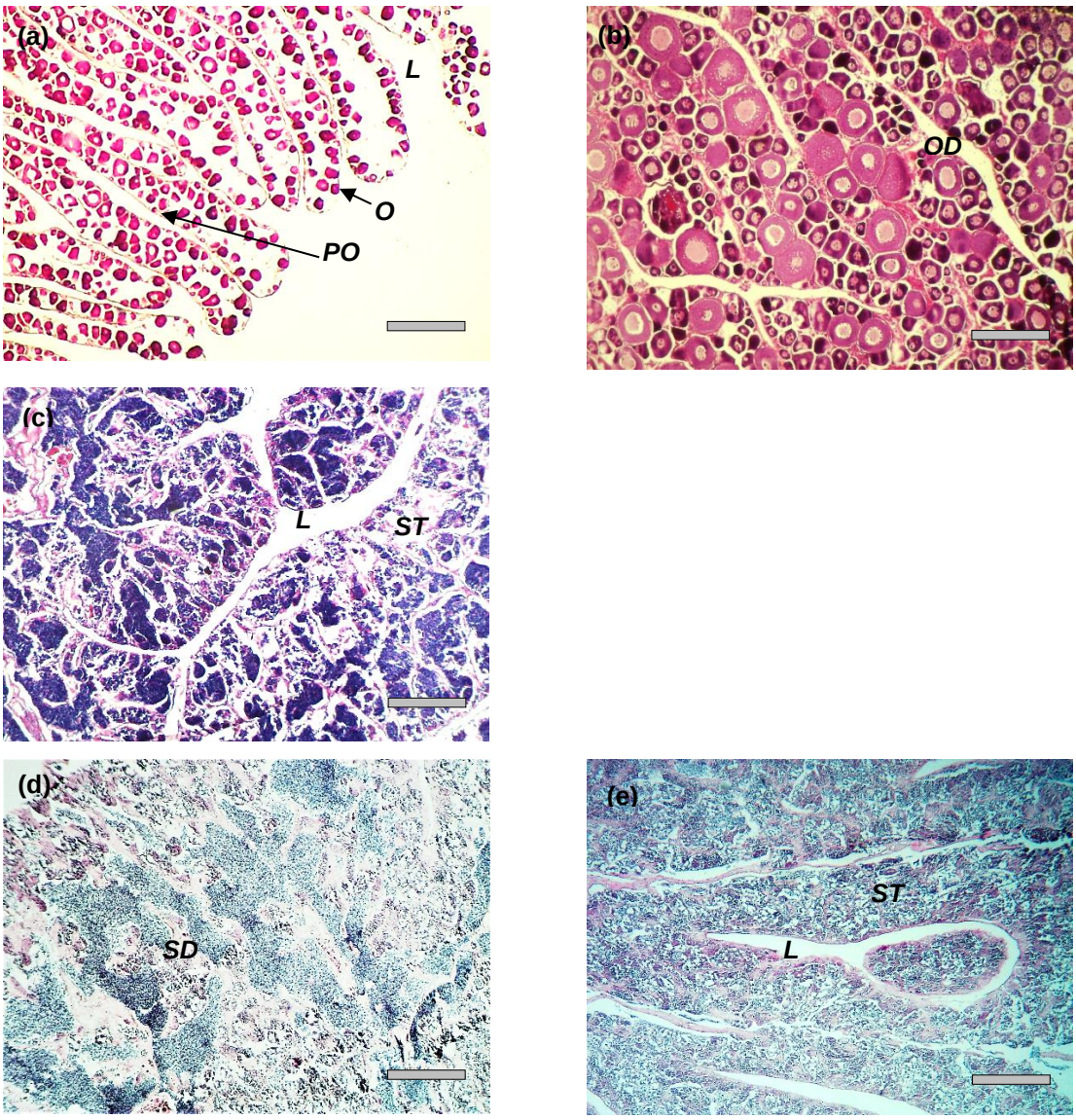

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535 **Figure 4.**

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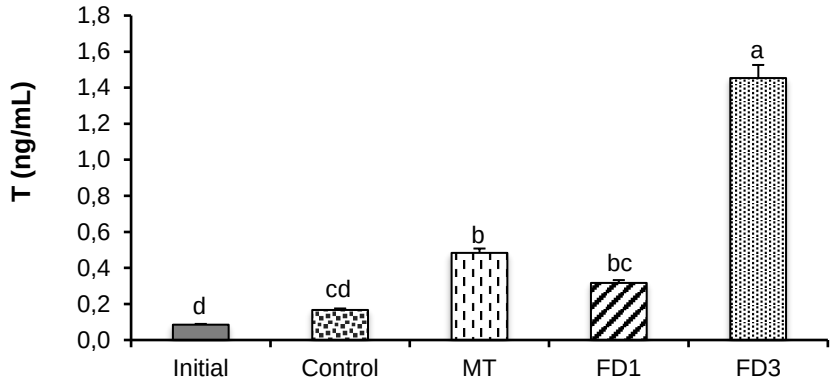

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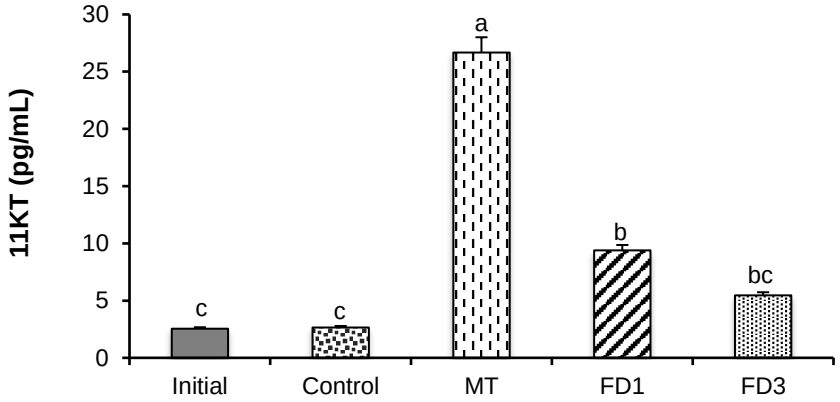

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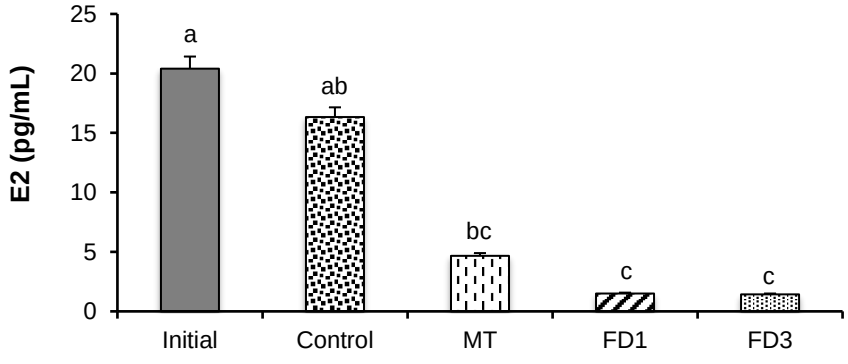

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542 **Figure 5.**

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