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Artificial masculinization in tilapia involves androgen receptor activation

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ABSTRACT

Estrogens have a pivotal role in natural female sexual differentiation of tilapia while lack of steroids results in testicular development. Despite the fact that androgens do not participate in natural sex differentiation, synthetic androgens, mainly 17-α-methyltestosterone (MT) are effective in the production of all-male fish in aquaculture. The sex inversion potency of synthetic androgens may arise from their androgenic activity or else as inhibitors of aromatase activity. The current study is an attempt to differentiate between the two alleged activities in order to evaluate their contribution to the sex inversion process and aid the search for novel sex inversion agents. In the present study, MT inhibited aromatase activity, when applied in vitro as did the non-aromatizable androgen dihydrotestosterone (DHT). In comparison, exposure to fadrozole, a specific aromatase inhibitor, was considerably more effective. Androgenic activity of MT was evaluated by exposure of *Sciaenochromis fryeri* fry to the substance and testing for the appearance of blue color. Flutamide, an androgen antagonist, administered concomitantly with MT, reduced the appearance of the blue color and the sex inversion potency of MT in a dose-dependent manner. In tilapia, administration of MT, fadrozole or DHT resulted in efficient sex inversion while flutamide reduced the sex inversion potency of all three compounds. In the case of MT and DHT the decrease in sex inversion efficiency caused by flutamide is most likely due to the direct blocking of the androgen binding to its cognate receptor. The negative effect of flutamide on the efficiency of the fadrozole treatment may indicate that the masculinizing activity of fadrozole may be attributed to excess, un-aromatized, androgens accumulated in the differentiating gonad. The present study shows that when androgen receptors are blocked, there is a reduction in the efficiency of sex inversion treatments. Our results suggest that in contrast to natural sex differentiation, during sex inversion treatments, androgens, either endogenous or exogenous, participate in inducing testicular differentiation.

1. Introduction

Rearing monosex, all-male population of fish is highly desirable in certain species in aquaculture due to higher growth rates of males, better coloration or as a mean of controlling unwanted reproduction (reviewed by Pandian and Sheela, 1995). This practice is especially important in the culture of tilapia in which males grow 20% faster than females and where uncontrolled breeding may severely damage profits of aquaculture operations. A few methods are currently available for producing all-male populations including manipulations of ambient temperature (Baroiller and D'Cotta, 2001; D'Cotta et al., 2001b,a) or hybridizations (Devlin and Nagahama, 2002). One of the methods widely used is the administration of synthetic androgens as masculinizing agents. The most common androgen employed in sex-reversal procedures is 17-α-methyltestosterone (MT), being effective in over 25 species examined (Pandian and Sheela, 1995). Although the efficiency of the treatment cannot be denied, it is potentially hazardous for both humans and the environment. Effluents originating in treatment ponds may leach into natural and drinking waters and affect consumers and wildlife. Extended exposure to androgens is associated with menstrual irregularities, virilization, atrophy of the breasts, and clitoromegaly in women; and impotence, premature cardiovascular disease and prostatic hypertrophy in men. Acne, baldness and cardiovascular and hepatic damage occur in both genders (Ferner, 1994). Governmental regulations (European, 2003) and increasing public concern regarding the use of hormones in the husbandry of animals cultured for human consumption call for finding an environmentally friendly substitute for inducing sex reversal in tilapia.

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In tilapia, a gonochoristic species, the first histological signs of sexual differentiation, are apparent at around 23–26 days post fertilization (dpf) (Kwon et al., 2001; Ijiri et al., 2008). It is accepted that endogenous estrogens, produced by cytochrome P450 aromatase, act as ovarian inducers, driving the undifferentiated gonad to develop into an ovary (Guiguen et al., 1999, 2010; Nakamura et al., 2003). In tilapia, ovarian aromatase gene expression shows a distinct sexual dimorphism apparent from 11 to 27 dpf (10 days prior to histological differentiation): Aromatase gene expression in males is much lower than in females, indicating an important role for estrogens in tilapia sex determination (Kwon et al., 2001; Ijiri et al., 2008). Inhibition of aromatase activity in developing fry by oral administration of fadrozole (a specific, non-steroidal aromatase inhibitor) induces complete sex inversion, causing genetic females to differentiate into males (Kwon et al., 2000, 2001; Afonso et al., 2001; Kobayashi et al., 2003). Ovarian development can be induced by exposing the fry to estrogens during the critical period of sexual differentiation, further establishing estrogens as key factors in sexual differentiation in tilapia (Chang et al., 1995; Nakamura et al., 1998; Guiguen et al., 1999; Kobayashi et al., 2003). Since 11-β hydroxylase, the enzyme responsible for the production of 11-ketotestosterone (11-KT) is absent in the male gonad during the critical period of sexual determination it was suggested that endogenous androgens are not responsible for testicular differentiation (Nakamura et al., 2003; Ijiri et al., 2008).

MT is a potent androgen as its binding to the androgen receptor is much stronger than that of endogenous natural androgens and of non-steroidal androgen antagonists such as flutamide (Fitzpatrick et al., 1994). Exposure to MT was found to suppress aromatase expression throughout sex differentiation, culminating in complete masculinization of genetically female tilapia fry (Bhandari et al., 2006). The activity of synthetic androgens as masculinizing agents is unexpected as androgens do not seem to take part in natural testicular differentiation. The sex inversion properties of MT and dihydrotestosterone (DHT) may be exerted by inhibition of aromatase activity as suggested by Mor et al. (2001), directly reducing estrogen levels in the differentiating gonad, or by binding to the androgen receptor, or both. In the current study we attempted to differentiate between the two activities in order to evaluate their relative contribution to the process of sex inversion.

In contrast, the sex inversion activity of aromatase inhibitors can easily be explained in the context of the critical role that estrogens play during natural sex differentiation. However, as aromatase uses androgens as a substrate, its inhibition may cause elevated levels of un-aromatized androgens that may affect the gonad.

This research is aimed at investigating the roles androgens, both endogenous and exogenous, play during induced sex inversion treatments.

2. Materials and methods

2.1. Aromatase activity assay

Ovarian microsomes were used to evaluate aromatase activity in vitro. Ovarian microsomes were prepared according to Guengerich and Hayes (1982). Briefly, tilapia ovaries were homogenized on ice using a glass-Teflon homogenizer in KCl 0.15% (w/v). The homogenate was centrifuged at 10,000*g* for 20 min, and the supernatant was centrifuged at 100,000*g* for 1 h at 4 °C. The resulting microsomal pellet was dissolved in phosphate buffer (0.1 M K_2HPO_4, 1 mM EDTA, pH = 7.4), homogenized and centrifuged at 100,000*g* for 1 h at 4 °C. The microsomal pellet was dissolved in a minimal amount (50 μl/100 mg tissue) of storage buffer (10 mM TRIS–HCl, 1 mM EDTA, 20% v/v glycerol, pH = 7.4) and homogenized again. Microsomes were stored at −80 °C until use.

In vitro inhibition of aromatase activity was tested using the tritiated water assay adjusted from Gonzalez and Piferrer (2002). The assay is based on the cleavage of the labeled hydrogen from the 1β-[^{3}H]-androstenedione used as substrate. The resulting tritiated water (^3H_2O) was measured and used as a parameter of aromatase activity. Unless otherwise stated, all reagents were purchased from Sigma. Five microliters of microsomal preparation were incubated for 6 h at 27 °C with continuous shaking in 200 μl phosphate buffer (10 mM K_2HPO_4, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, pH = 7.4) containing 10 nM 1β-[^{3}H]-androstenedione (Perkin Elmer, Waltham, MA, USA) and 1.5 mM NADPH. Reaction was stopped by the addition of 1 ml of ethyl ether followed by vortex mixing. Ether and aqueous layers were allowed to separate for 10 min, and then transferred to −80 °C for 15 min to freeze the aqueous layer. The organic phase was discarded and the aqueous phase was mixed with 2 volumes of dextran coated charcoal slurry (5% w/v coated charcoal in phosphate buffer) and centrifuged for 5 min at 3000*g* at 4 °C to precipitate charcoal and substrate. The aqueous phase (300 μl) was collected and mixed with 5 ml scintillation fluid (Quicksafe A, Zinsser analytic, Frankfurt, Germany). β-Radiation was determined in a Canberra-Packard Tri-Crab 1600CA β-counter (Canberra Industries Inc., Meriden. CT, USA). Each treatment was performed in triplicate and experiments were repeated at least three times.

2.2. Fish and experimental design

All experimental procedures were in compliance with the Animal Care and Use Guidelines at the Hebrew University and approved by the local Administrative Panel on Laboratory Animal Care Committee.

For both fish species handling of eggs and fry was carried out in a similar manner. Following natural fertilization eggs were gently extracted from the female's oral cavity and incubated in down-welling incubation jars until complete absorption of the yolk sac. Free swimming fry (age 11 days post fertilization (dpf)) were then housed in 120 L plastic tanks for feeding trials at a density of 2 fish/L. Temperature of 27 ± 1 °C was maintained at all times by submersed heaters (Jager, 100 W, Germany). Commercial fish food (48% protein, 6% fat, Ra'anan Shivuk, Israel) was used for all trials. The test compounds were dissolved in ethanol and mixed with the feed. The mixture was allowed to dry at room temperature for 24 h and then stored at 4 °C. Fry were fed ad libitum for 12 h each day using automatic feeders. Fry were fed with the test compounds for 4 weeks, after which feeding commenced with untreated diet for 6 more weeks until fish were large enough for sexing.

In this study we employed a novel in vivo model for the detection of androgenic response, namely, the bright blue coloration in males of a common ornamental fish species, *Sciaenochromis fryeri*. In this species, the males naturally attain a bright blue coloration at the onset of sexual maturity (around the age of 6 months) while females and young fry are silvery gray. This secondary sex characteristic can be induced artificially if *S. fryeri* fry females are exposed to androgens. This treatment causes these normally gray fish, to develop blue coloration within 2 weeks, allowing easy identification of androgenic effect. We utilized this unique characteristic to test for MT activity at the androgen receptor by feeding MT to fry in the absence and presence of different levels of the specific androgen receptor antagonist flutamide.

S. fryeri fry were obtained from a commercial breeder at an age of 7 days post fertilization. In order to test for MT activity at the androgen receptor, MT was fed to fry in the absence and presence of different levels of the specific androgen receptor antagonist

flutamide (0, 500, 1000, 2000 or 5000 mg/kg food; 25 fish in each group). MT was used at 60 mg/kg food, which is the concentration used in aquaculture for sex inversion of tilapia.

After 3 weeks of feeding, fry were examined for the appearance of the typical blue color. At the age of 4 months fish ($n = 20$) were sexed using the gonad squash method (Guerrero and Shelton, 1974).

Flutamide is a nonsteroidal androgen antagonist with no androgenic or estrogenic activity that acts by blocking androgen receptor sites and preventing translocation of the hormone receptor complex. It was found efficient in blocking the androgenic effects of DHT in vivo (Meaney et al., 1983; Poyet and Labrie, 1985).

Tilapia (*Oreochromis niloticus*) were bred in our fish facility. Fry were obtained by mating either a XX or YY male with a normal female to produce either a monosex female or a monosex male population respectively. Seven treatments were tested on XX fry: Control; MT 60 mg/kg food; MT 60 mg/kg food + flutamide 5000 mg/kg food; DHT 60 mg/kg food; DHT 60 mg/kg food + flutamide 5000 mg/kg food; fadrozole 250 mg/kg food (according to Kobayashi et al., 2003) and fadrozole 250 mg/kg food + flutamide 5000 mg/kg food. Tilapia XY fry were fed either a control diet or flutamide 5000 mg/kg food. No significant differences were found in the weight of the fry between treatment groups during the experimental feeding period (data not shown) implying that reduced feed intake cannot account for the differences in sex ratios. Sexing of 50 juvenile fish from each treatment was performed by the gonadal squash method (Guerrero and Shelton, 1974).

2.3. Statistical analysis

The results of each treatment are expressed as means ± SEM. Statistical analyses were performed using Prism 4 (Graph-Pad Software, San Diego, CA, USA). The significance of differences between group means was determined by one-way analysis of variance (ANOVA) followed by Newman–Keuls test using the same software. Different letters mark data sets that are significantly different. EC_{50} values were calculated from concentration–response curves using computerized nonlinear curve-fitting using the same software. Color intensity scoring was performed using ImageJ by conversion of the blue component into gray values and combining the blue area and pixel intensity measurements in each fish ($n = 6$). For sexing experiments, χ^2 analysis was performed for every pair of treatments using JMP 5 software (SAS Institute, Version 5.1, Cary, NC, USA). Significance was imparted at the $p < 0.05$ level.

3. Results

3.1. In vitro aromatase inhibition

When incubated with ovarian microsomes, both MT and fadrozole showed a substantial ability to inhibit tritiated water production from 1β-[^{3}H]-androstenedione used as a substrate. MT showed significant inhibition ($p < 0.01$) at doses as low as 150 nM. DHT, a non-aromatizable androgen, showed an inhibition trend similar to that exerted by MT. Fadrozole showed a much stronger inhibition with an EC_{50} stronger by more than two orders of magnitude. Flutamide and E_2 had no effect on aromatase activity (Fig. 1).

3.2. Determination of the effective flutamide dose

In order to determine the effective dose of flutamide needed to inhibit the androgenic activity of MT, *S. fryeri* fry were fed food containing MT (60 mg/kg) concomitantly with graded doses of flutamide (Fig. 2). A bright blue tint was apparent in fish fed MT alone after 3 weeks of feeding. Flutamide fed concomitantly with MT reduced the appearance of the blue color in a dose dependent manner. Nevertheless, even the highest concentration of flutamide (5000 mg/kg food) did not completely eliminate the appearance of blue color. Fish fed the control diet remained grey.

3.3. Effect of treatments on sex ratio

In *S. fryeri* fry fed graded doses of flutamide concomitantly with MT the male ratio in the population decreased in a dose dependent manner in parallel with the flutamide dose. *S. fryeri* fry fed with MT + flutamide 5000 mg/kg exhibited a sex ratio similar to that of the control group (Fig. 3).

Treatment of genetically female tilapia (*O. niloticus*) fry (beginning at 11 dpf) with MT (60 mg/kg food) or fadrozole (250 mg/kg feed) for 28 days resulted in complete masculinization of the fish (100% males). Flutamide (5000 mg/kg food) fed concomitantly with MT to genetically female tilapia was able to inhibit its masculinizing activity, resulting in 69% females. When fed at 60 mg/kg food, DHT showed a much weaker sex inversion potency compared to MT with only 12% of treated fry developing testes. Flutamide fed concomitantly with DHT completely blocked its potency producing 100% females as in the untreated control. Flutamide administered with fadrozole reduced its sex inversion efficiency resulting in 21% females. Although 5% females were identified in flutamide treated XY fish, flutamide alone did not significantly alter ($p = 0.27$) the sex ratio of XY tilapia fry (Fig. 4).

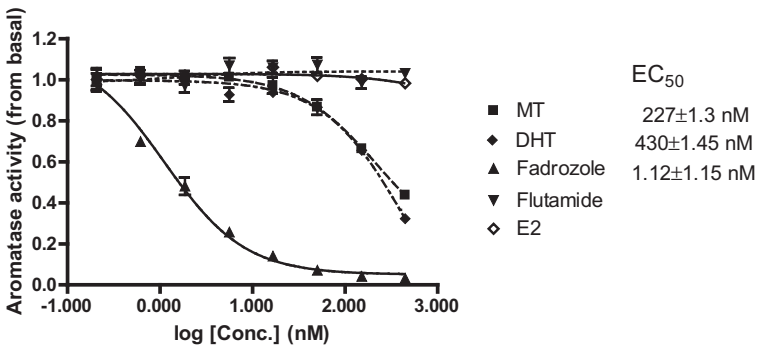

Fig. 1. Effect of 17-α-methyltestosterone, flutamide, DHT, E_2 and fadrozole on aromatase activity in tilapia ovarian microsome preparation. Aromatase activity is expressed relative to basal activity (tritiated water production in the absence of MT, DHT, E_2, fadrozole or flutamide) ± SEM. EC_{50} values were calculated from concentration–response curves using computerized nonlinear curve-fitting.

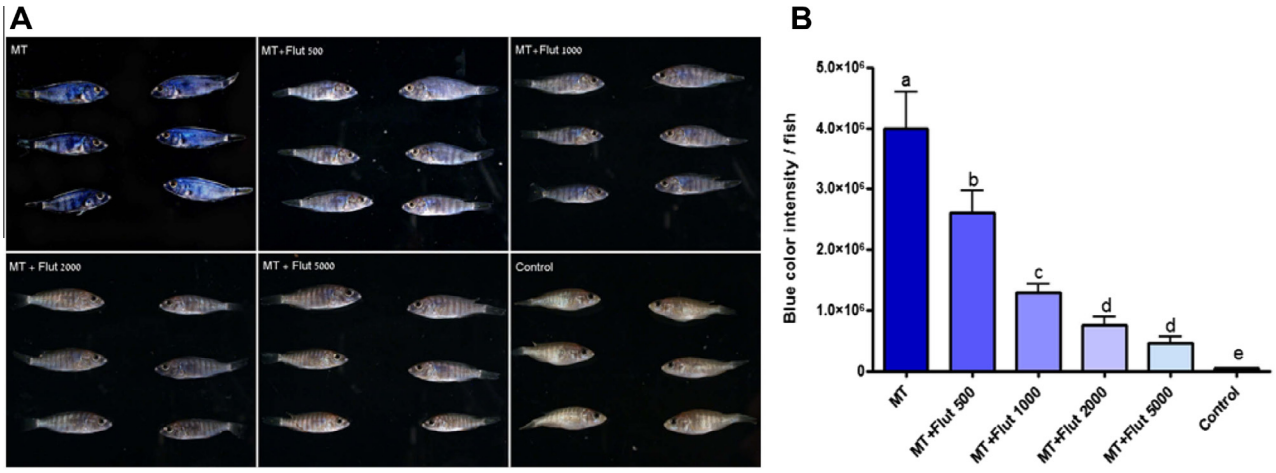

Fig. 2. (A) Development of blue color in *Sciaenochromis fryeri* fry fed for 3 weeks with food containing MT (60 mg/kg food); MT (60 mg/kg food) + flutamide 500 mg/kg; MT (60 mg/kg food) + flutamide 1000 mg/kg; MT (60 mg/kg food) + flutamide 2000 mg/kg; MT(60 mg/kg food) + flutamide 5000 mg/kg food; fish fed food containing no additives (control) failed to develop blue coloration. (B) Quantification of the blue color intensity of the fish exposed to the different treatments.

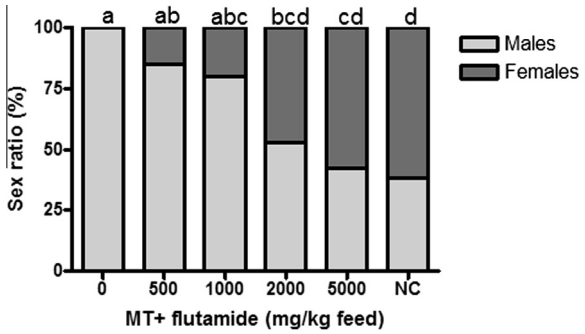

Fig. 3. Sex ratio of *Sciaenochromis fryeri* ($n = 25$) fry fed increasing doses of flutamide concomitantly with MT (60 mg/kg food). 0: MT 60 mg/kg food; NC (Negative control): fry fed with untreated food. Different letters represent treatments that are significantly different ($p < 0.05$).

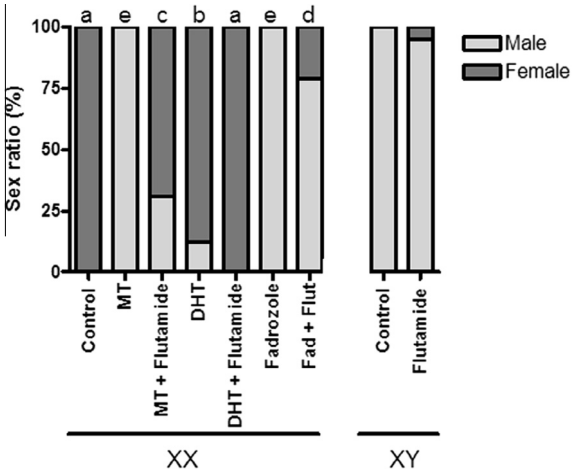

Fig. 4. Effects of different treatments on sex ratio of tilapia fry ($n = 50$). Genetically female (XX) fry were treated with 7 different diets: Control; MT 60 mg/kg food; MT 60 mg/kg food + flutamide 5000 mg/kg food; DHT 60 mg/kg food; DHT 60 mg/kg food + flutamide 5000 mg/kg food; fadrozole 250 mg/kg food or fadrozole 250 mg/kg food + flutamide 5000 mg/kg food. Genetically male (XY) tilapia fry were fed either a control diet or a diet containing flutamide at 5000 mg/kg food. Different letters represent treatments that are significantly different ($p < 0.05$).

4. Discussion

For years, synthetic androgens, predominantly MT, have been used as sex inversion agents for the production of all-male populations of fish. Although very effective, the mechanism underlying the masculinizing effect of MT has not been fully clarified and serious concerns and regulations exist regarding its safety. The current study attempted to cast light on this mechanism as a mean of focusing the search for alternative compounds.

In order to establish the correct flutamide dose for blocking the androgenic activity of MT an assay using color development as a secondary sexual male characteristic was used. Male secondary sexual characteristics are associated with high androgen levels (Ankley et al., 2001; Bayley et al., 2002; Panter et al., 2004). Several in vivo assays for evaluating androgenic effects have been developed in fish including use of color, sperm count, gonopodium development and courtship behavior in guppies (Bayley et al., 2002), and reproductive development and induction of nuptial tubercules in fathead minnows (Ankley et al., 2001; Panter et al., 2004). Most assays concentrate on short term effects of compounds and do not test for functional sex inversion. More recently, assays measuring spiggin production by kidneys of three-spined sticklebacks both in vivo (Katsiadaki et al., 2006) and in vitro (Jolly et al., 2006), were introduced for testing androgenic activity of different substances. Unfortunately, although in vitro trials are extremely efficient at screening potential activities of different compounds, they can not accurately predict the compound's activity in vivo due to differential uptake, degradation and cell penetration in the organism. In the current study, a simple in vivo assay was used based on color changes in *S. fryeri* fry. MT caused a significant color change in the fry. Flutamide, an androgen receptor antagonist, was able to decrease the androgenic activity of MT in vivo in a dose dependent manner, although even at concentration 80 times higher than MT, the appearance of the blue color was not completely eliminated. This could be due to a weaker affinity of flutamide, compared to MT, to the androgen receptor (Fang et al., 2003) or to its inferior ability to reach the target tissues when administered orally.

Using *S. fryeri* as a bioassay enables us to test not only for the transient masculinizing effect of different substances, but also allows us to test their effect on sex differentiation and functional sex inversion in a species phylogenetically related to tilapia. Our findings suggest that the androgenic activity of MT is responsible

for its sex inversion activity in *S. fryeri* since flutamide was able to successfully inhibit the androgenic activities of MT, seen as a decrease in the intensity of the blue color appearance in *S. fryeri* fry accompanied by a decrease in sex inversion efficiency.

MT was found to competitively inhibit aromatase activity in a cell line (Mor et al., 2001). Part of the inhibitory effect of MT observed in the in vitro system, may be due to aromatization of MT to 17-α-methylestradiol, a functional estrogen, as described by de Gooyer et al. (2003) and Hornung et al. (2004). However, DHT, a non-aromatizable androgen, also showed an inhibitory effect, very similar in its pattern to that of MT, indicating that androgens can effectively inhibit aromatase activity and supporting the assumption that the inhibition of tritiated water production by MT may be attributed to inhibition of aromatase activity. When compared to fadrozole, a specific non-steroidal aromatase inhibitor, we found MT to be significantly less potent in inhibiting tritiated water production in vitro.

Despite exhibiting similar aromatase inhibition effects, our results show that in tilapia, DHT is significantly less effective than MT in inducing sex inversion, although its binding affinity to the androgen receptor is considerably stronger than that of MT (Fang et al., 2003). This is in accordance with the combined results of Asahina et al. (1989), Davis et al. (1992), Piferrer et al. (1993) who found DHT to be less potent than 11-KT which in turn is less effective than MT in inducing sex inversion.

As apparent in both species of fish used in this study, when the androgenic activities of MT and DHT are blocked, their ability to achieve complete sex inversion is reduced, suggesting that the androgenic activities of these compounds are responsible for their activities as sex inversion agents. This finding implies that MT and DHT exert their masculinizing activity through activation of the androgen receptor rather than as direct inhibitors of aromatase activity. Surprisingly, flutamide also reduced the sex inversion efficiency of the aromatase inhibitor fadrozole. This suggests that androgens, probably a result of unprocessed testosterone substrate, play an important role in fadrozole-induced sex inversion. Since at the time of sexual differentiation both testosterone synthesis enzymes and androgen receptors are present in XX tilapia fry (Ijiri et al., 2008), unprocessed androgens can activate receptors, thus making the shift towards testicular differentiation. Moreover, during induced sex inversion in rainbow trout aromatase inhibitor treatment caused an elevation of steroidogenic enzyme expression (Vizziano et al., 2008). This finding supports the possibility of excess, un-aromatized androgens that are able to affect the differentiating gonad during aromatase inhibitor treatments. The notion of a common, androgen driven, sex inversion response is further supported by the findings of other groups that both androgen and aromatase inhibitor treatments cause shared gene responses during the masculinizing treatments, including a reduction in *cyp19a* gene expression (Kitano et al., 2000; Vizziano et al., 2008; Navarro-Martin et al., 2009).

It is widely accepted that during natural sex differentiation estrogens are responsible for ovarian differentiation while lack of estrogens induces testis development (Guiguen et al., 1999). Our findings, suggest that artificial masculinization in tilapia does not follow exactly the same pathways operating in natural sex differentiation. While in natural sex differentiation androgens do not appear to play an important role, during artificial sex inversion, androgens, either from an exogenous or an endogenous source, play a significant role in inducing testicular differentiation.

The fact that flutamide by itself did not significantly alter sex ratio in tilapia is consistent with the findings of Nakamura et al. (2003) that the lack of estrogens and not the presence of androgens is essential for testicular differentiation. This also explains the failure of using flutamide and other androgen antagonists in feminization treatments (Hagino et al., 2001; Chardard et al., 2003). However, in the present study 5% females were found in the flutamide treatments (compared to 0% in the control) suggesting that further investigation is necessary to fully understand the role endogenous androgens play during sex differentiation.

In conclusion our findings suggest that androgens, either endogenous or exogenous, play a pivotal role in artificial/induced sex inversion. When searching for novel sex inversion agents, both androgen receptor activators and aromatase inhibitors should be considered.

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