

Use of 17- α -methyltestosterone for production of male secondary sexual characteristics in the adult female green swordtail (*Xiphophorus hellerii*)

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Abstract. The green swordtail *X. hellerii* Heckel, 1848 is a commercially important ornamental species. As phenotypic males with the sword extension of the caudal fin are more valuable economically than fish lacking the sword, masculinization by means of 17- α -Methyltestosterone (MT) was tested. Populations of female broodstocks were fed MT-treated feeds (20, 35 and 50 mg MT kg⁻¹ feed) at a rate of 50 g of MT-treated feed per kilogram of fish biomass (5% body weight [BW]) for 45 consecutive days to cause development of male secondary sexual characteristics, specifically the growth of the sword extension. Use of MT feed at 5% BW for 45 d resulted in 100% masculinization, whereas 0% masculinization occurred in green swordtails fed control feed. There were no significant differences between MT-treated groups in the proportion of phenotypic males. Also we found no significant differences in mortality among experimental groups. No degeneration of swords was seen 200 d after the last day of experimental feed treatment. According to our finding, the use of MT-treated feeds can increase the production of higher-value phenotypic male green swordtails and enhance profitability for aquaculturists.

Keywords: green swordtail (*X. hellerii*), phenotypic male, 17- α -methyltestosterone, sex reversal, masculinization.

چکیده (In Persian). دم شمشیری، *X. hellerii* یک گونه زینتی مهم تجاری است. نظر به اینکه نرهای فنوتیپی با باله دمی کشیده شمشیری، نسبت به ماهی فاقد دم شمشیر مانند ارزش اقتصادی بیشتری دارند، نرسازی به وسیله هورمون ۱۷-آلفا-متیل تستوسترون (MT) آزمایش شد. تعدادی از مولدین ماده با غذاهای تیمار شده با MT (۲۰، ۳۰، ۵۰ میلی گرم MT بر کیلوگرم غذا) با نرخ ۵۰ گرم غذای تیمار شده با MT به ازای هر کیلوگرم وزن ماهی (۵٪ وزن بدن [BW]) برای ۴۵ روز متوالی تغذیه شدند، تا باعث ایجاد صفات ثانویه جنسی نر، مخصوصاً رشد شمشیر کشیده شود. استفاده از MT با نرخ پنج درصد وزن بدن برای ۴۵ روز باعث ۱۰۰ درصد نرسازی شد، درحالیکه در دم شمشیری های تغذیه شده با غذای کنترل هیچگونه نرسازی رخ نداد. هیچگونه اختلاف معنی داری بین گروه های تیمار شده با MT در نسبت نرهای فنوتیپی وجود نداشت. همچنین ما دریافتیم که اختلاف معنی داری در مرگ و میر بین گروه های آزمایش وجود ندارد. ۲۰۰ روز پس از آخرین روز تیمار غذای آزمایشی هیچگونه تحلیل در شمشیر مشاهده نشد. بر طبق یافته های ما، استفاده از غذای تیمار شده MT می تواند تولید دم شمشیری های نر فنوتیپی با ارزش تر را افزایش دهد و سودبخشی آبری پروری را افزایش دهد.

کلمات کلیدی: دم شمشیری (*X. hellerii*)، نرهای فنوتیپی، ۱۷-آلفا-متیل تستوسترون، تغییر جنسیت،

نرسازی

Rezumat. Peștele xifo verde, *X. hellerii*, este o specie ornamentală importantă din punct de vedere comercial. Deoarece indivizii fenotipic masculi, care prezintă o extensie a radiilor înotoătoare caudale (spadă), sunt mai valoroși din punct de vedere economic decât femelele (fără spadă), noi am testat masculinizarea loturilor prin administrare de 17- α -metiltestosteron (MT). Populațiile de femele reproducătoare au fost hrănite cu furaj tratat cu MT (în doze de 20, 35 and 50 mg MT kg⁻¹ furaj) concentrație estimată la 50 g de furaj tratat cu MT per kilogram biomasă pește (5% din greutatea corporală [BW]) pe durata a 45 zile consecutive, în vederea inducerii caracterelor sexuale secundare masculine, mai precis a spadei. Utilizarea furajului cu MT 5% BW timp de 45 d s-a concretizat în 100% masculinizare, în timp de martorul a înregistrat un procent de masculinizare de 0%. Nu au existat diferențe semnificative între grupurile experimentale tratate cu MT în ceea ce privește proporția indivizilor fenotipic masculi. De asemenea, nu s-au constatat diferențe semnificative de mortalitate între loturile experimentale. Nici un caz de degenerare a spadei nu a fost observat după 200 d de la ultima zi experimentală. Conform acestor cercetări, utilizarea furajului cu MT poate crește proporția de masculi fenotipici și profitabilitatea acvăriștilor.

Cuvinte cheie: Xifo verde (*X. hellerii*), masculi fenotipici, 17- α -metiltestosteron, inversiune sexuală, masculinizare.

Introduction. Sex determination and differentiation in different species of fish have been shown to be influenced by a wide variety of factors, including genetics (e.g., monogenic versus polygenic systems, if present, and autosomal factors); environment (e.g., temperature and pH); behavioral and physiological mechanisms (e.g., social sex-switching in the protandrous clownfishes *Amphiprion* spp.); xenobiotics; gonochoristic versus hermaphroditic status; and hormone release and levels (Devlin & Nagahama 2002). Timing and relative influence of different factors during ontogeny can greatly alter sexual differentiation and development and the corresponding presence of secondary sexual characteristics (Devlin & Nagahama 2002). Even in aquacultured species that have been in production for decades, many of these factors have not been completely identified.

The green swordtail *X. hellerii* is a commercially important aquacultured ornamental species. Phenotypic males (i.e., with sword extensions of the caudal fin) can be worth as much as twice the value of phenotypic females (i.e., fish lacking swords).

Swordtails *Xiphophorus* spp. are indistinguishable by sex until approximately 3–9 weeks of age, when the anal fin starts to develop into a gonopodium in males (except late-maturing males). During this period of sexual maturation, males also become more laterally compressed and develop the sword extension on the ventral portion of the caudal fin (Weaver 1998). In one study, 35–45% of green swordtail males were mature by 15 weeks (50–52 mm caudal length [CL]: length from snout to posterior portion of caudal fin, not including the sword), whereas by 28 weeks 96–100% of males (59–61 mm CL) were mature (Weaver 1998).

Studies of sex determination in green swordtails have suggested that in addition to potential genetic factors, environmental factors including temperature and pH may influence sex ratios in a population (Rubin 1985; Devlin & Nagahama 2002).

Use of hormones, specifically methyltestosterone and related steroids, has proven effective for masculinization of fish in a number of species. Consequently, we evaluated the androgen 17- α -methyltestosterone (MT) for use in green swordtails to cause the expression of the caudal sword, a male secondary sexual characteristic of primary importance in the ornamental fish industry. Various studies in the literature document the efficacy of the use of MT in green swordtails for encouraging secondary male sexual characteristics (Lim et al 1992; Nava-Bautista & Rodriguez-Gutierrez 1997; Jessy & Varghese 1988), but these studies have been primarily conducted on fry or juveniles. Oral administration of MT to 28-d-old fry at the rate of 35 mg MT kg⁻¹ feed for 40 d resulted in 100% masculinization and brighter coloration by the middle of the treatment period (Nava-Bautista & Rodriguez-Gutierrez 1997). Three-day-old fry fed at a rate of 35 mg MT kg⁻¹ feed four times per day for 40 d had 96.8% masculinization by the end of the treatment period (Nava-Bautista & Rodriguez-Gutierrez 1997). Also use of 80, 100, 120, and 140 mg MT kg⁻¹ feed in green swordtails resulted in 100% masculinization (Jessy & Varghese 1988). In another study, 28-d-old green swordtails fed either 500 or 750 μ g MT g⁻¹ body weight (BW) on alternate days over a period of 10 d had 100% masculinization and demonstrated no significant enhancement or suppression of growth (Lim et al 1992).

However recently, Yanong et al (2006) demonstrated that adult female green swordtails fed by MT-treated feeds at dose 60 mg MT kg⁻¹ feed for 28 d resulted in 100% masculinization and no regression of swords was seen 28 d after the experimental feeding period.

Typical production practices of green swordtails involve stocking outdoor ponds with broodstock and setting traps to harvest market-size fish after several months. This trapping and marketing can continue for 6–18 months. Males stop growing after reaching sexual maturity (Kallman 1983), and males that reach sexual maturity prior to achieving market size are collected on a continuous basis. In addition, sex ratios in some populations of swordtails have been shown to be normally skewed toward females (Snelson 1989). Therefore, the ratio of marketable males to females continues to decline throughout the production cycle.

The objective of present study was to assess the efficacy of MT, when administered by feed, for the development of male secondary sexual characteristics (in particular, a caudal sword) in adult phenotypic female green swordtails and posttreatment changes. This study was planned to determine how to achieve maximum treatment efficiency, i.e. what are the minimum doses of MT and minimum periods of time that would result in 100% masculinization in the adult female green swordtails.

Materials and Methods. Experimental fish were phenotypic females of 4.7 cm in length and 6 months of age which were obtained from the private production ponds in Kashan region. Prior to experiment, the fish were allowed to acclimate for 4 weeks in tanks located inside the Fishery Laboratory at the Isfahan University of Technology.

After acclimation, at the start of the trial the mean weight (\pm SE) for all fish in each tank were weighed in bulk (1.65 ± 0.06 g) and all fish were examined again for the presence or absence of a caudal sword.

Water changes of 95% were made once daily. Tanks were siphoned after first feeding each day (see below), and subsequently, water was added from dechlorinated water source and was allowed to fill each tank to approximately 60 liters.

Ten fish were randomly assigned to each of the twelve 60-L tanks (Table 1). Tanks were covered with glass covers, and were supplied with continuous air pumping. The aquaria system was housed inside an experimental room with natural photoperiod (12 h light and 12 h dark). Water quality was checked at the beginning of the trial and 2 d week⁻¹ in randomly selected tanks throughout the duration of the experiment. Total ammonia nitrogen, nitrite nitrogen, and pH were measured by use of a Fish Farmers Test Kit (Hach Co., Loveland, Colorado); temperature and dissolved oxygen were tested with a WTW OXI 196 Dissolved Oxygen Meter.

Feeds was made from Starter Feed of Trout after grinding and sieving as SFT (size-00) that had nutrient analyses similar to those of feeds used in the ornamental aquaculture industry. The prepared diet was a fine meal-like feed that floats on the surface and then gradually sinks over time.

Tanks were randomly assigned to one of four feed treatments. Methyltestosterone was added to MT-treated feeds through a pure ethanol spray carrier. The ethanol volatilizes rapidly from the feed, and only the MT remains. There were one control feeds and three MT-treated feeds. MT treated feeds contained 20, 35 and 50 mg MT kg⁻¹ feed, and control feeds lacked MT. MT-treated feeds was made of 1 mg MT l⁻¹ ethanol stock solution and Control feeds were sprayed only with ethanol. The food was then mixed well and left to dry in the sun for several hours. All feeds were properly labeled and stored in a standard freezer at -18°C. As a precautionary measurement, gloves were worn when handling feed, water, or fish throughout this experiment.

All fish were acclimated to control diets for 14 d prior to the start of the experiment. Subsequently, fish were fed experimental diets at a rate of 5% of BW once daily for 45 d; Fish were checked daily for mortality. The expression of caudal swords was measured on days 0, 23 and 46 during experiment. Also the fish from each tank were weighed to determine the effect of each treatment on the growth. Combined fish weight for each tank and feed weight was obtained by use of a calibrated scale (SHIMADSU AEU-210). In addition, the presence or absence of swords was checked by

inspection of each fish on day 200 after the last day of experimental MT treatment and expression of caudal swords and fish weight was measured. The experiment was a completely randomized design with three treatment groups and one control group, each containing three replicate tanks (4 experimental groups \times 3 replicates = 12 tanks) and each tank containing ten fish.

Table 1

Feed treatments used in green swordtail masculinization trials wherein 17- α -methyltestosterone (MT) was added to feed to produce caudal swords in phenotypic females (control = untreated feed; BW = body weight). Three replicate tanks were used for each treatment. Treated feeds were administered for 46 d, and fish were observed for another 200 d to check for sword regression

<i>Treatment</i>	<i>Feed type</i>	<i>Dosage*</i>	<i>%BW fed</i>
1	MT	35	5
2	Control	0	5
3	MT	20	5
4	MT	50	5

*mg MT kg⁻¹ feed

Data files were entered into Excel (Microsoft Corp) spreadsheets and were imported into SPSS software for statistical analysis. Fish weight data (total weight of fish in tank) and caudal sword growth data were analyzed by use of one-way analysis of variance (ANOVA) followed by Duncan. Mortality data were analyzed by means of a Chi-Square Test. A type I error rate (α) of 0.05 was used for all analyses.

Results and Discussion. Water quality measurements for the experiment were as follows (mean \pm SD): temperature was 23.5 \pm 0.5°C; dissolved oxygen was 6.5 \pm 0.55 mg L⁻¹; total ammonia was 1.2 \pm 0.56 mg L⁻¹; nitrite was 0.03 \pm 0.04 mg L⁻¹ and pH was 7.3 \pm 0.

The administration of MT in feed according to the regimens described in this experiment (20, 35 and 50 mg MT kg⁻¹ feed, fed at a daily rate of 5% BW, for 45 d) was 100% effective in producing the desired male secondary sexual characteristic (the sword caudal extension) preferred in the industry (Figure 1).



Figure 1. Photograph of a green swordtail (after treatment with 17- α -methyltestosterone [MT] in feed) on day 46 of an experiment to determine the effect of MT administration on the development of male secondary sexual characteristics (i.e., development of a caudal sword) in phenotypic females. The MT-treated feed was administered for 45 d, after which the fish were observed for signs of sword regression (data in inches).

Based on qualitative observations of the tanks, sword development began in some treated fish by day 12 and swords were present in the majority of treated fish by day 16. Qualitatively, coloration was not noticeably different among treatment groups. Changes in lateral compression were not quantitatively determined; however from qualitative point of view, fish developed subtle changes in body shape. Thickening of anal fin rays led to partial or complete development of gonopodia. Inspection of individual fish revealed that all fish receiving MT treated feed had caudal swords (i.e., were phenotypic males) by the end of day 45, and no swords regressed during the 200 d after treatment. No control fish developed swords during the 45-d experiment. Given the fact that all experimental fish were individually examined, along with the 100% masculinization of treated fish and 0% masculinization of control fish, no statistical analysis was necessary to determine differences in MT treated versus control fish. Statistical analysis was indicated no significantly differences between MT-treated groups in the proportion of phenotypic males (Figure 2).

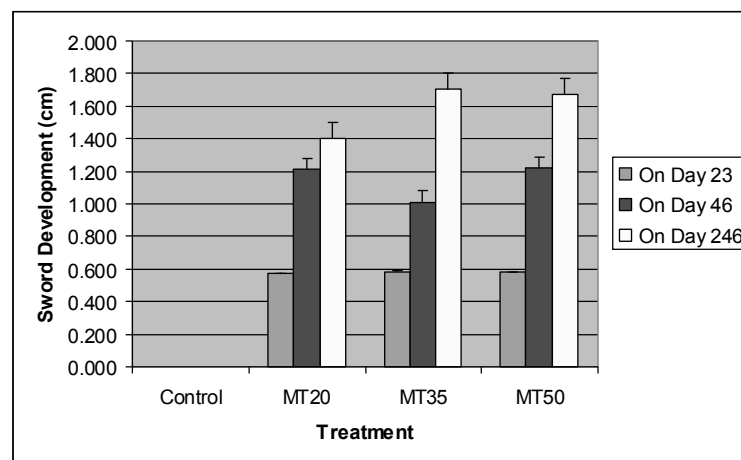


Figure 2. Sword development of a green swordtail with regards to different treatment with 17- α -methyltestosterone [MT] in feed on day 23, 46 and 200 days after termination hormonal treatment.

The regimens tested were safe with respect to survival and normal feeding and behavior for the duration of the experiment (the 46-d experimental feeding period and 200-d posttreatment observation period). There were no significant differences in mortality among any of the treatment and control groups (Chi-Square Test: $\chi^2 = 0.40$, $df = 3$, $P = 0.94$).

Although this experiment was originally designed only to examine statistical differences in sword development and mortality, weight gain was also compared. At the end of the treatment period on day 46 the mean weight gain (\pm SE) among experimental groups were as follows: $0.79 \pm .025$ g for the control group; 0.62 ± 0.07 g for the MT-treated group at 20 mg MT kg^{-1} feed, 0.71 ± 0.03 g for the MT-treated group at 35 mg MT kg^{-1} feed and, $0.44 \pm .083$ g for the MT-treated group at 50 mg MT kg^{-1} feed.

As is evident from our results, the highest dose of MT decreased weight gain. Figure 3 shows the mean weight gain of control fish was only significantly greater than that of MT-treated fish at 50 mg MT kg^{-1} feed ($p < 0.05$). However, no significant differences found among MT-treated fish groups (figure 2). Similarly, in immersion studies, Siamese fighting fish grew slower at higher doses of MT (Kavumpurath & Pandian 1994; Kirankumar & Pandian 2002). Also a greater increase in weight gain for the control group (lacked MT) than MT-treated group at 60 mg MT kg^{-1} feed was seen in Green swordtail (Yanong et al 2006). Growth advantages with androgen treatments have previously been reported in teleosts, possibly due to increased appetite and enhanced food utilization and protein synthesis (Jayaprakas & Sindhu 1996). However, a dose-dependent relationship was demonstrated in such growth responses in *Clarias batrachus* (Mukhopadhyay et al 1986). Given the fact that fish were late maturing males or adult females, reproductive status may have acted as a confounding factor for weight

differences among treatments. For example, condition (i.e., the relation of length and weight) can vary greatly according to reproductive status (Anderson & Neumann 1996). Indeed, males generally weigh less than females of similar length. Furthermore, the process of masculinization, with its diversion of energy toward the expression of secondary male characteristics, may in itself result in lower individual fish condition than that exhibited by female controls. Moreover, the green swordtails used in this experiment were adults and already of market size; therefore, any effect of MT on growth at this stage would not be important to production economics. In addition in posttreatment, on day 246 there were no significant differences in weights among any of the treatment and control groups.

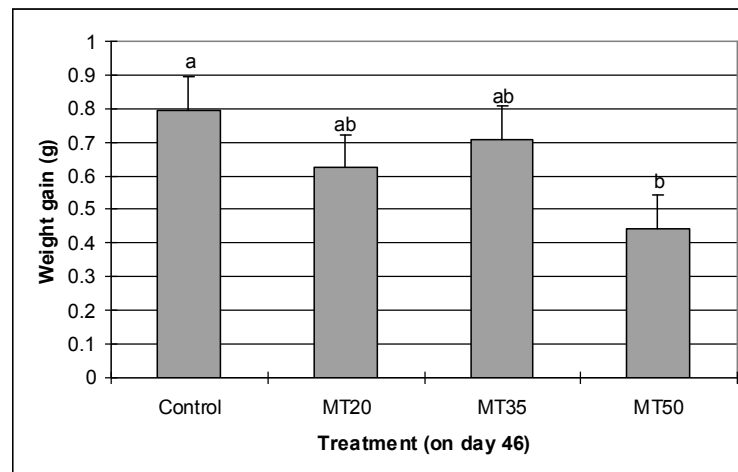


Figure 3. Effects of 17- α -methyltestosterone [MT] on Weight Gain of treated and untreated fish on day 46.

According to published papers, sex determination in swordtails is most likely polyfactorial, and specific determinants probably vary within the genus (Foerster & Anders 1977; Volff & Schartl 2001; Devlin & Nagahama 2002). In one study, a sex chromosome system was not detected for green swordtails but was observed for southern platyfish *X. maculatus* (Foerster & Anders 1977). One hypothesis holds that some swordtail species have a number of autosomal modifiers, each with different alleles of varying influence on the gonosomal sex determination gene (Kallman & Bao 1987). Another study suggested that environmental factors also affect sex determination. Specifically, low pH favored an increase in the proportion of males, although the study bears repeating owing to problems with experimental design (Rubin 1985). Although the green swordtail is considered to be a gonochoristic species, at least one report suggests that hermaphrodites exist, based on the observation of three individuals in one strain switching from female to male (Lodi 1979).

In conclusion, the short-term application of MT as a feed additive was effective in the expression of male secondary sexual characteristics; specifically, the development of the sword extension on the caudal fin; and no differences in mortality were observed between treated fish and controls; and no regression of swords during the 200 d after treatment during the observation period at the tested dosage rates for adult, market-size female green swordtails. Therefore, the use of MT-treated feeds can increase the production of higher-value phenotypic male green swordtails and could enhance profitability for aquaculturists. However, these fish presumably would not be very good breeders. Finally, according to our results, it is suggested to use lower doses of MT, since it is as effective as higher doses in development of male secondary sexual characteristics in adult phenotypic female.

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