

Full Length Research Paper

Masculinization of Swordtail *Xiphophorus hellerii* (Cyprinodontiformes: Poeciliidae) Treated with 17 α -Methyltestosterone and Vitamin E

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The green swordtail, *Xiphophorus hellerii*, is a commercially important ornamental species. Since phenotypic male with the sword extension of the caudal fin has more commercial value than female, masculinization by means of 17- α -methyltestosterone (MT) was tested. female brood stocks of swordtail were fed different dose rates of synthetic androgen MT (i.e. 25, 50 and 75 mg MT kg⁻¹) of feed and MT 25, 50 and 75 mg kg⁻¹ plus 30 mg kg⁻¹ Vitamin E. MT and vitamin (E+MT) were administrated orally by using pellet dry starter and ethanol alcohol investigated for 30 days. The fry also kept two weeks after feeding to maturation, at the end of experiment. Survival and mortality rate were determined. At the end of the experiment the sex ratio was determined by secondary sex characteristics and macroscopic examination of the gonads after dissection. The results of the present study showed that all MT receiving treatment showed a significantly higher male proportion than control ($p < 0.05$). Univariate Analysis of Variance (ANOVA) of survival rate showed that there is no significantly different among all experimental groups ($P > 0.05$). However, the survival rate of fries fed MT+vitamin E were higher in comparing with groups fed just MT. Results showed that dose rate of 75 mg MT kg⁻¹ of feed and Vitamin caused in maximum male population (98%) with 16.0% and 8.0% mortality, respectively.

Keywords: Swordtail, *Xiphophorus hellerii*, sex reversal, masculinization, vitamin E, survival.

INTRODUCTION

The androgen, 17 α -Methyltestosterone (MT), an anabolic steroid, is being widely used for masculinization of fish in a number of species (Amiri-Moghaddam et al, 2010; Kefi et al., 2013). Many species of ornamental fishes exhibit a marked sexual dimorphism due to the more pigmented bodies and larger fins usually observed in males that are preferred over the female fish by the hobbyists (Piferrer and Lim., 1997). As a result, the culture of all-male populations is a highly attractive approach. Green swordtail (*Xiphophorus hellerii* Heckel, 1848) is a commercially important ornamental fish species (Amiri-Moghaddam et al., 2010) that 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) (Yanong -

et al., 2006).

Because the most valuable and commercially important swordtails, broadly studied in terms of masculinization using MT; masculinization by oral administration of feed incorporated with methyltestosterone is the most effective and practical method for the production of all male swordtail (Ortega-Salas et al., 2013). The dosage rates vary from 10-100 mg MT kg⁻¹ of diet for swordtail. Sex reversed swordtail showed a better growth rates than normal (Karayucel et al., 2006). In addition, the survival rates by different level of MT depend on the duration and dose of hormone. Nava-Bautista and Rodriguez-Gutierrez (1997) reported oral administration of MT to 28-day old fries at the rate of 35 mg MT kg⁻¹ feed for 40 day which resulted in 100%

masculinization and brighter coloration by the middle of the treatment period. Also, they stated 3-day old fries 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. Jessy and Varghese (1988) reported use of 80, 100, 120, and 140 mg MT kg feed in green swordtails resulted in 100% masculinization. Lim et al. (1992) stated 28-day old swordtails fed either 500 or 750 µg MT g body weight on alternate days over a period of 10 d had 100% masculinization and demonstrated no significant enhancement or suppression of growth. Yanong et al (2006) demonstrated that adult female green swordtails fed by MT 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. Amiri-Moghaddam et al. (2010) indicated that female broodstocks of swordtails were fed MT-treated feeds at a rate of 50 g of MT-treated feed per kilogram of fish biomass (5% body weight [BW]) for 45 days resulted in 100% masculinization. Also, Mousavi-Sabet and Ghasemnezhad (2013) used 5, 10, 20, 40, 80 and 200 mg of MT kg⁻¹ for swordtails and stated 40 mg MT kg⁻¹ of feed resulted in maximum male population with 14.17±3.97% loss of fish while 200 mg MT kg⁻¹ of feed showed 41.67±5.15% mortality.

Since, there are numerous factors involve such as genetics, environment, behavioral and physiological mechanisms, xenobiotics, gonochoristic, hermaphroditic status, hormone release and levels that often influence efficacy; the results have shown somewhat variable (Devlin and Nagahama, 2002). Nutrient supplementation in fish diets has been an economically promising method for improving the performance of different intensive fish production systems (Ispr et al., 2011). Vitamin E is among the most important nutrients which influence the fish immune system, and the supply of vitamin E can reduce mortality and improve fish performance, while increasing specific and nonspecific immune responses (Puangkaew et al., 2004). In addition, vitamin E is a potent antioxidant that offers protection against oxidative damage to various fish tissues (Adham et al., 2000).

Considering the above mentioned facts, the objective of this study was to determine the efficacy of different levels of 17α-methyltestosterone to find out optimum dose for masculinization of swordtail and evaluating the potential of vitamin E in survival of swordtail during masculinization.

MATERIALS AND METHODS

Experimental fish

Swordtail were phenotypic females which used in this study has obtained from RVK ornamental fishes Breeding and Culture Company in Iran. Prior to experiment, the fish were allowed to acclimate for 2

weeks in aquarium located inside the Fishery Laboratory. All of the swordtail fries used in these studies was obtained from these broodstocks.

Fries rearing conditions

Prior to the experiment, for acclimation of new born swordtails to the laboratory conditions, they fed by nauplius *Artamia salina* for 6 days. A total of 480 fries were placed in 24 glass aquaria and each aquarium contained 50 L of freshwater that was continuously aerated with a 5-cm air stone and filtered by a normal sponge filter. 95% water volume daily changed and water was added from dechlorinated water source. Also, siphoned faeces and other particles were extracted out from the bottom of each aquarium after each feeding. The aquarium was equipped with continues aeration system and was filled up to a culture volume of 50 L. The important water quality parameters was checked and recorded every day and kept at 28±1°C, oxygen concentration above 5.5 mg/L and pH (7.5± 0.3). 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 (name of company and country). The aquaria systems were housed inside an experimental room with natural photoperiod (12 h light and 12 h dark). Fish were checked daily for mortality.

Preparation of hormone treated diet

The fries were fed daily with a commercial diet (dry starter pellet food with 40% crude protein), six times per day, for 30 days (20% body weight [BW]). A stock solution of hormone 17α-methyltestosterone was prepared by dissolving the steroid in Ethanol (96⁰) at a concentration of 1mg ml⁻¹ and the solution sprayed weighed amount of feed. Even distribution of the chosen hormone was ensured by stirring thoroughly during spraying by synthetic hormones MT was used from Aboreihan Pharmacy Company. For preparation of vitamin E we used Alcohol Evaporation Method.

The experimental design was tested by RCBD (Randomized Complete Block Design). At the end of experiment, the head and tail of the swordtail were cut off and the body fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin sectioned at (5µm thicknesses) and stained with eosin-haematoxylin for histological examination. The tissues were observed under microscope 40X powers. Tissues were recorded as gonads.

Experimental designs

The experiment was a completely randomized design

Table 1. Sex composition of green swordtail fed diets treated with varying doses of 17- α -methyltestosterone (MT) and vitamin E for 30 days duration and results of survival rate.

Experimental group	Male (%)	Female (%)	Survival (%)
CG1 (Control group)	45.0	55.0	88.0
CG2 (Ethanol group)	50.0	50.0	87.0
A (25 mg MT kg ⁻¹)	46.0	54.0	84.0
AE (25 mg MT kg ⁻¹ + 30 mg VitE kg ⁻¹)	42.0	58.0	84.0
B (50 mg MT kg ⁻¹)	72.0	28.0	84.0
BE (50 mg MT kg ⁻¹ + 30 mg VitE kg ⁻¹)	78.0	22.0	85.0
C (75 mg MT kg ⁻¹)	98.0	2.0	84.0
CE (75 mg MT kg ⁻¹ + 30 mg VitE kg ⁻¹)	98.0	2.0	92.0

with six treatment groups and two control group, each containing three replicate tanks (8 experimental groups * 3 replicates = 24 tanks) and each tank containing 20 fish. The experiment groups were defined as follows:

- 180 fry division in 9 glass aquarium for 30 days and they introduced to 3 different level of MT i.e. 25, 50 and 75 mg MT kg⁻¹ of feed, so in this manner we named their A, B and C experimental groups, respectively.
- In other experiment, 180 fry division in 9 glass aquarium for 30 days were introduced to 3 different level of MT i.e. 25, 50 and 75 mg MT kg⁻¹ of feed plus 30 mg vitamin E in per kg of feed, so in this manner we named their AE, BE and CE experimental groups, respectively.
- Two Control Group with three replicates inclusive: CG1= this group did not receive any hormone and vitamin E and CG2 or ethanol group control= this group were fed with a diet treated with ethanol.

Statistical analysis

Data were entered into Microsoft Excel (ver. 2010) and then imported into SPSS software (ver. 12) for statistical analysis. The statistical analysis of the means of masculinization between control groups and treated groups analyzed by Univariate analysis of variance (ANOVA) and followed by Duncan's Multiple Range (DMR) test. Logistic model regression was used to analyze the data of sex reversal. These models were used to study such type of experimental group where there is a binary response, Y=1 (male in this case), Y=0 (not male), which may be influenced by explanatory variables. Mortality data were analyzed by Heterogeneity chi-square tests. I error rate (α) of 0.05 was used for all analyses.

RESULTS

In the first step we tried to found out the minimum dose of 17 α -methyltestosterone to obtain all male population

of swordtails. Results of this study showed that minimum male population (98%) was recorded for dose 75 mg kg⁻¹ MT of feed for 30 days (group C). The dose of 50 mg kg⁻¹ MT of feed for 30 days resulted in 72% males (group B). Statistical analysis of sex reversal data 25, 50 and 75 mg kg⁻¹ MT showed a significant difference ($P \leq 0.05$) among all the two sexes (male, female under all the treatments) (fig. 1). The results of the present study showed that all MT receiving treatments had a significantly higher male proportion than controls except dose 25 mg kg⁻¹ MT (group A) and dose 25 mg kg⁻¹ MT with vitamin E (group AE). percentage of male population in each group was; 45% in control group (CG1); 50% in ethanol group (CG2); in A and AE experimental groups 46% and 42%, respectively; in dose B and BE experimental groups 72% and 78%, respectively; in C and CE experimental groups 98% and 98%, respectively (Table1). Percentage of male between normal doses and doses with vitamin E did not show any significant different, so we conclude that use of vitamin E does not have any effect in masculinization.

The logistic regression obtained from the treatment by 17 α -methyltestosterone in A, B, C, AE, BE and CE experimental groups and controls of present study remained as:

$$\text{Logit}(\mu) = (65 \times 10^{-5}) X^2 - (0.378)$$

Logit (μ) = occurrence of male

X= hormone dose

The survival rate of control (CG1, CG2) and all off experimental groups of fry of hormone treated varied between 84% and 92% in the 30 days of experiment (Table1). Univariate statistics (ANOVA) of survival rate showed that there were not significantly different among all experimental groups ($P > 0.05$). However, the survival rate of swordtail fries fed vitamin E were higher in comparing with groups fed just on different level of MT. Results of this study showed that minimum mortality (8%) was recorded for CE group. Survival rate which observed in the experiment groups and controls showed in figure 1.

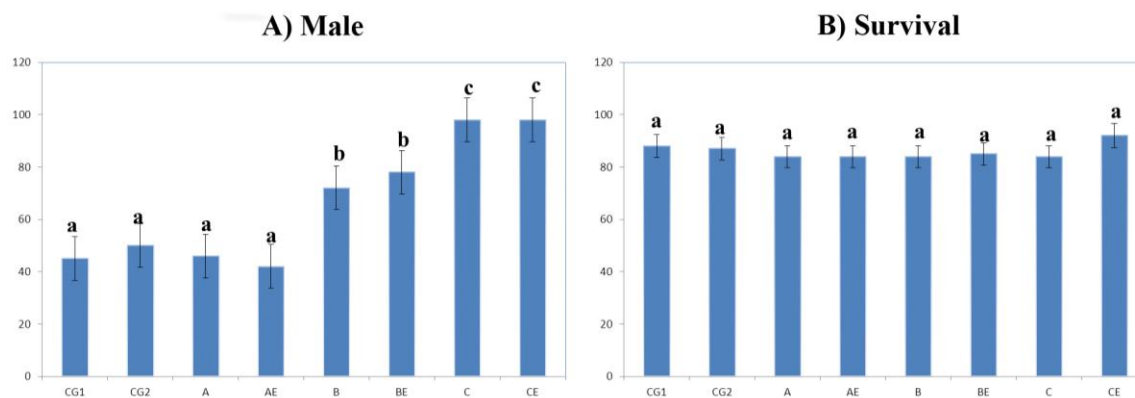


Figure 1. Mean percentage of male (A) and survival rate (B) in each treatment groups of green swordtail fed diets treated with varying doses of 17- α -methyltestosterone (MT) and Vitamin E for 30 days duration.

DISCUSSION

Our results demonstrated that oral administrations of MT to swordtail can result in 98% masculinization by 75 mg MT kg⁻¹ of feed for 30 days. Also, logistic regression obtained from the treatment by 17 α -methyltestosterone in different group shown masculinisation in swordtail was dose dependent and with increasing concentrations of MT caused an increasing in the number of males.

Sex determination in swordtails is most likely poly factorial, and specific determinants probably vary within the genus (Voff and Schartl, 2001; Devlin and Nagahama 2002). Kallman and Bao, (1987) stated some swordtail species have a number of autosomal modifiers, each with different alleles of varying influence on the gonosomal sex determination gene. Rubin (1985) stated environmental factors specifically low pH favored an increase in the proportion of males affect sex determination. The efficacy of a variety of chemicals when applied to water is affected by water quality. Alkalinity total hardness and pH often alter the efficacy or toxicity of chemical dissolved in water (Rubin, 1985).

Low survival of fish treated with steroid hormones were reported in general a treatment involving steroid hormone results in higher mortality of some species such as *Salmo salar* (Sower et al., 1984) and *Barbus conchoniis* (Karayucel et al., 2006). Mousavi-Sabet and Ghasemnezhad (2013) reported increasing concentrations of MT caused an increasing in mortality of swordtail. Andersen et al. (2006) reported short-term exposure of male zebrafish to the lowest concentration of methyltestosterone (4.5ng MT L⁻¹) increased VTG synthesis. In the some experiment high mortality of MT could be resulted from aromatization (the catalysing procedure converting androgen to estrogens) of MT to estrogens stimulating high vitellogenin production which damage liver of fish (Karayucel et al., 2006). In this study the survival rate of swordtail fry fed on different level of

MT were higher than the control groups. Higher mortality in some group perhaps is related to exhaustion subsequence to enhanced liver metabolism, since it is well known that exposure to estrogenic compounds strongly induced vitellogenin production, which enlarges the liver (Karayucel et al., 2006). However, the results of this survey clearly showed that survival rate of swordtail fry fed vitamin E were higher in comparing with the control groups and groups fed just on different level of MT. Therefore, vitamin E had beneficial effects on the survival rate of swordtail but there was not observe significant different among groups. In the other hand, adequate vitamin E supplementation in fish diets under intensive rearing is essential for survival and growth performance (Nekoubin et al., 2012). Naziroglu et al. (2003) mentioned that vitamin E has very effective role on immune system response and it is one the few nutrients for which supplementation with higher than the recommended levels enhance certain aspects of immune function in fish.

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 of the caudal fin. Furthermore, the vitamin E added to this feed significantly increased the survival rate. However, more investigation is required to understand the role of vitamin E in biological processes which do not necessarily involve its antioxidant function.

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