

Effect of dietary administration of methyltestosterone and vitamin C on the sex reversal and survival of *Xiphophorus maculatus* (Cyprinodontiformes: Poeciliidae)

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Abstract. The common platy, *Xiphophorus maculatus*, is a commercially important ornamental species. Since phenotypic male has more commercial value than female, masculinization by means of 17- α -methyltestosterone (MT) was tested. Three-five (3-5) day post-hatch larvae were fed by 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 100 mg kg⁻¹ Vitamin C (Vit C). MT and vitamin were administrated orally by using pellet dry starter and ethanol alcohol investigated for 45 days. 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. Univariate Analysis of Variance (ANOVA) of masculinization was significant ($p < 0.05$) however results of Duncan test showed that there is no significant difference among all experimental groups, in addition with intensification in MT dose, the masculinization was increased. Results of addition Vit C to treatments was significant ($p < 0.05$) and results of Duncan test showed that there is significant difference among all experimental groups however in higher dose of MT survival was lower than low dose of MT. Vit C increased survival rate in groups 25 and 50 mg MT kg⁻¹. Nevertheless the group 75 mg MT kg⁻¹+Vit C had lower survival rate that this group without Vit C. The obtained results showed that dose rate of 75 mg MT kg⁻¹+Vit C caused maximum male population (55.96%) with 17.81% mortality, and dose rate of 25 mg MT kg⁻¹ without Vit C caused minimum male population (49.47%) with 12.62% mortality. In conclusion results showed the doses of MT used in this study were not effective to masculinize the common platy fishes and Vit C was not able to increase survival in high dose of hormone.

Key Words: common platy, *Xiphophorus maculatus*, sex reversal, masculinization, vitamin C.

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; Faghani-Langroudi et al 2014; Mousavi-Sabet 2011). Some species of ornamental fishes exhibit a marked sexual dimorphism due to more pigmented bodies and larger fins in males that are preferred over the female fish by the hobbyists (Piferrer & Lim 1997; Mousavi-Sabet & Ghasemnezhad 2013). As a result, the culture of all-male populations is a highly attractive approach (Khiabani et al 2014; Mousavi-Sabet et al 2012). Poeciliids, including the livebearers, are found in fresh and brackish waters from the eastern United States south through the Caribbean to northeastern Argentina and in Africa (Nelson 2006; Mousavi-Sabet & Eagderi 2014). The platy fish, *Xiphophorus maculatus*, is a commercially important ornamental fish species that belongs to the family Poeciliidae. These small freshwater fish (approximately 5 cm), are viviparous, present variable body coloration (Jordan 1963), and are distributed from Southern United States down to South America (Nelson 2006). Because the most valuable and commercially important Poeciliidae family, 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 fishes (Amiri-Moghaddam et al 2010; Mousavi-Sabet & Ghasemnezhad 2013; Ortega-Salas et al 2013; Khiabani et al 2014). The dosage rates are varied for example in swordtail (*Xiphophorus hellerii*) from 10-100 mg MT kg⁻¹ of diet (Khiabani et al 2014). 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 & Rodriquez-Gutierrez (1997) reported oral administration of MT to 28-day old fries at the rate of 35 mg MT kg⁻¹ feed for 40 days 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 days had 96.8% masculinization by the end of the treatment period. Jessy & Varghese (1988) reported use of 80, 100, 120, and 140 mg MT kg⁻¹ 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 days 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 days resulted in 100% masculinization and no regression of swords was seen 28 days 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 kg of fish biomass (5% body weight [BW]) for 45 days resulted in 100% masculinization. Also, Mousavi-Sabet & Ghasemnezhad (2013) used 5, 10, 20, 40, 80 and 200 mg of MT kg⁻¹ for swordtails and showed that 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 involved in masculinization 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 & Nagahama 2002). Nutrient supplementation in fish diets has been an economically promising method for improving the performance of different intensive fish production systems (Mousavi-Sabet 2007; Ispir et al 2011). Vitamin C (ascorbic acid, AA) plays a key role for normal physiological functions in fish (Lim & Lovell 1978). Since most teleosts are unable to synthesize ascorbic acid due to the lack of L-gulonolactone oxidase, an exogenous source of vitamin C is required in fish diets. The existence of a need for a dietary supply of ascorbic acid for growth and development has evidenced in a number of fish (NRC 1993). Inadequate supply of dietary vitamin C usually resulted in a number of deficiency symptoms such as reduction in growth, skeletal deformities, impaired collagen formation and hemorrhaging (Halver et al 1969; Gouillou-Coustans et al 1998). However, the availability of this vitamin increases growth, survival, stress resistance and etc. (Mousavi-Sabet et al 2013; Mousavi-Sabet et al 2015). 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 common platy and evaluating the potential of vitamin C in survival of common platy during masculinization.

Material and Method

Experimental fish. The platy fish female broodstocks which used in this study were obtained from RVK ornamental fishes' breeding and culture Company in Iran. Broodstocks were allowed to acclimate for 1 week in aquarium located inside the Fishery Laboratory. All of the platy fish fries used in these studies were obtained from these broodstocks and were 3-5 days post-hatch.

Fries rearing conditions. A total of 540 fries were placed in 27 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. Fifty percent (50%) water volume was replaced daily and fresh water was added from dechlorinated water source. Also, siphoned faeces and other particles were removed out from the bottom of each aquarium

after each feeding. Each aquarium was equipped with continuous aeration system and was filled up to a culture volume of 50 L. The important water quality parameters were checked and recorded every day. Temperature kept at $27\pm 1^{\circ}\text{C}$, oxygen concentration above 5.5 mg L^{-1} and pH (7.5 ± 0.3). Total ammonia nitrogen, nitrite nitrogen and pH were measured by using a Fish Farmer Test Kit (Hach Co., Loveland, Colorado); temperature and dissolved oxygen were tested by a WTW OXI 196 Dissolved Oxygen Meter. 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 by a commercial diet (dry starter pellet food with 40% crude protein), six times per day, for 45 days (20% body weight [BW]). A stock solution of hormone 17 α -methyltestosterone was prepared by dissolving the steroid in Ethanol (96 $^{\circ}$) at a concentration of 1 mg 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 C, Alcohol Evaporation Method was used.

Experimental designs. The experiment was a completely randomized design with six treatment groups and three control groups, each containing three replicate tanks (9 experimental groups * 3 replicates = 27 tanks) and each tank containing 20 fish. The experiment groups were defined as follows:

1. One hundred and eighty (180) fry were divided in 9 glass aquaria for 45 days and were introduced to 3 different levels of MT i.e. 25, 50 and 75 mg MT kg^{-1} of feed, so in this manner we named them as T1, T3 and T5 experimental groups, respectively;

2. In other experiments, 180 fries were divided in 9 glass aquaria for 45 days and were introduced to 3 different levels of MT i.e. 25, 50 and 75 mg MT kg^{-1} of feed plus 100 mg vitamin C (Vit C) per kg of feed, so in this manner we named them as T2, T4 and T6 experimental groups, respectively;

3. three control groups with three replicates include: control group (CG): this group did not receive any hormone, alcohol and Vit C; ethanol group control (EG): this group were fed by a diet treated with ethanol and no hormone or Vit C; and vitamin group (VG): this group were fed by a diet treated with Vit C and no hormone or alcohol.

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.

Statistical analysis. Data were entered into Microsoft Excel (ver. 2010) and then imported into SPSS software (ver. 16) 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 and Discussion. Results showed that the doses of MT used in this study were not enough effective to masculinize the common platy. Univariate analysis of variance (ANOVA) of masculinization was significant ($p < 0.05$) (Table 1), however results of Duncan test showed that there is no significant difference among all experimental groups, in addition with intensification in MT dose, the masculinization was increased (Figure 1).

Table 1
ANOVA results of survival and masculinization in platy fish fed by diet containing MT and Vit C

		Sum of squares	df	Mean square	F	Sig.
Survival	Between groups	353.00	8	44.00	21.00	0.00
	Within groups	37.00	18	2.07		
	Total	390.00	26			
Masculinization	Between groups	6.00	8	0.00	0.00	0.00
	Within groups	74.00	18	4.00		
	Total	80.00	26			

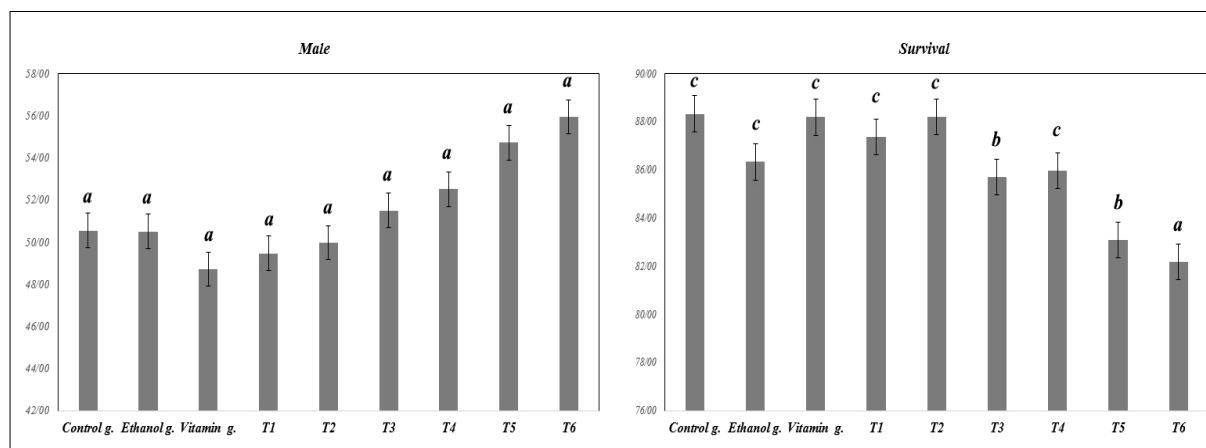


Figure 1. Mean percentage of male (left) and survival rate (right) in each treatment groups of platy fish fed by diets treated with varying doses of MT and Vit C for 45 days.

The results of the present study showed that dose rate of 75 mg MT kg⁻¹+Vit C (T6) caused maximum male population (55.96%) with 17.81% mortality, and dose rate of 25 mg MT kg⁻¹ without Vit C (T1) caused minimum male population (49.47%) with 12.62% mortality. Percentage of male population in each group was; 50.56% in CG, 50.51% in EG and 48.71% in VG; in T1, T2 and T3 experimental groups 49.47%, 49.97% and 51.50%, respectively; in T4, T5 and T6 experimental groups 52.52%, 54.72% and 55.96%, respectively (Table 2). Percentage of male between normal doses and doses with Vit C did not show any significant different, so we conclude that use of Vit C does not have any effect in masculinization (Figure 1).

Table 2
Sex composition and survival rate of platy fish fed by diets treated with varying doses of MT and Vit C for 45 days

Experimental group	Male (%)	Female (%)	Survival (%)
Control group (0 mg MT kg ⁻¹ + 0 mg VitC kg ⁻¹ +no ethanol)	50.56	49.44	88.33
Ethanol group (0 mg MT kg ⁻¹ + 0 mg VitC kg ⁻¹ +with ethanol)	50.51	49.49	86.33
Vitamin group (0 mg MT kg ⁻¹ + 100 mg VitC kg ⁻¹ +no ethanol)	48.71	51.29	88.19
T1 (25 mg MT kg ⁻¹ + 0 mg VitC kg ⁻¹)	49.47	50.53	87.38
T2 (25 mg MT kg ⁻¹ + 100 mg VitC kg ⁻¹)	49.97	50.03	88.19
T3 (50 mg MT kg ⁻¹ + 0 mg VitC kg ⁻¹)	51.50	48.50	85.71
T4 (50 mg MT kg ⁻¹ + 100 mg VitC kg ⁻¹)	52.52	47.48	85.97
T5 (75 mg MT kg ⁻¹ + 0 mg VitC kg ⁻¹)	54.72	45.28	83.09
T6 (75 mg MT kg ⁻¹ + 100 mg VitC kg ⁻¹)	55.96	44.04	82.19

The survival rate of controls (CG, EG and VG) and fry of hormone treated varied between 83% and 88% in the 45 days of experiment (Table 2). Results of Vit C showed that Vit C was not able to increase survival in high dose of hormone. However in higher dose of MT survival was lower than low dose of MT and Vit C increase survival rate in groups 25 and 50 mg MT kg⁻¹ with Vit C (T2 and T4) than these groups without Vit C. Nevertheless the group 75 mg MT kg⁻¹ + Vit C (T6) had lower survival rate than this group without Vit C. Results showed that minimum and maximum mortality were recorded for AC and CC group, respectively. Survival rate which observed in the experiment groups and controls showed in Table 2.

The logistic regression obtained from the treatment by 17 α -methyltestosterone in T1-T6 experimental groups and controls of present study remained as:

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

where: Logit (μ) = occurrence of male;
X = hormone dose.

Our results demonstrated that oral administrations of MT to platy fish cannot result in masculinization but it may increase survival for 45 days. Also, logistic regression obtained from the treatment by 17 α -methyltestosterone in different group showed masculinization in platy was dose dependent and with increasing concentrations of MT caused an increasing in the number of males.

Sex determination in Poeciliidae is most likely poly factorial, and specific determinants probably vary within the genus (Volf & Schartl 2001; Devlin & Nagahama 2002). Kallman & 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 by 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 & 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 (*Danio rerio*) to the lowest concentration of methyltestosterone (4.5 mg MT L⁻¹) increased VTG synthesis. In 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 platy fish fries feed on different level of MT were lower 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; Khiabani et al 2014). However, the results of this survey clearly showed that survival rate of platy fish fries fed Vit C were lower in comparing with the control groups and groups fed just on different level of MT. Therefore, Vit C had not beneficial effects on the survival rate of fries. There are different factors effect on growth rate and pattern in Poeciliids e.g., temperature, sexuality, diet, supplements and etc. (Mousavi-Sabet et al 2014; Eagderi et al 2015; Moshayedi et al 2015a; Moshayedi et al 2015b). Felix (1989) assessed the relative growth promoting efficiency of MT on platy and stated that the hormone MT has promoted the growth rate to a significant level over the control. The enhanced growth rate, appetite and food consumption were obtained in *Cyprinus carpio* (Lone & Matty 1980), *Oncorhynchus mykiss* (Ostrowski & Garling 1986), *Carassius auratus* (Yamazaki 1976), *Oncorhynchus kisutch* (Fagerlund et al 1979), and *Channa striata* (Nirmala & Pandian 1983) using MT.

In fishes, it has been established that Vit C plays an important role in fish reproduction (Blom & Dabrowski 1995; Emata et al 2000; Dabrowski & Ciereszko 2001). Vit C was known to increase reproduction performance such as ovarian growth in *C. carpio* (Watanabe & Takashima 1977), higher percentage of normal eggs and fecundity in gilthead sea bream, *Sparus aurata* (Izquierdo et al 2001). Diets deficient in Vit C resulted in high mortality of eyestalk-ablated *Penaeus japonicus* females (Alava et al 1993a) retarded the ovarian development of *P. japonicus* (Alava et al 1993b) and decreased the hatching rate of *Penaeus indicus* eggs (Cahu et al 1995). The results of this study showed that Vit C supplemented diets improved the fry survival (%) in groups 25 and 50 mg MT kg⁻¹ but not in 75 mg MT kg⁻¹. These revealed that vitamin C incorporated diets helped to increase the survival of the experimental fish. Dabrowski & Ciereszko (2001) reported that Vit C is necessary for successful reproduction in *O. mykiss*, apparently in protecting oxidation sensitive genetic material in gametes and probably has the same function in warm-water fish.

Conclusions. The short term application of MT as a feed additive was not effective in the expression of male secondary sexual characteristics. Furthermore, the Vit C added to this feed was not able to increase survival in high dose of hormone. However, more investigation is required to understand the role of Vit C in biological processes which do not necessarily involve its antioxidant function.

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