Research Article

Masculinization of Flowerhorn by Immersion in Androgens

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Received May 28, 2008; Accepted November 6, 2008

Abstract

This research objective was to investigate the effects of two synthetic androgens, 17 α-methyltestosterone (MT) and mesterolone, on sex reversal in flowerhorn fish by hormonal immersion with the aim of introducing a new method for producing all-male flowerhorn progeny. In this study, 6 days post-hatched fry (swim-up stage) were immersed with either MT or mesterolone at different concentrations (125, 250 or 500 μgL^{-1}). One hundred and forty swim-up fry were allocated to each of 21 100-L aquaria. Experimental fishes were exposed to hormone for 24 hours before 10 L of water was added and the first water change (approximately 80%) was done on day 5. Fish were reared until they reached the size that histological examinations of gonad can be performed (4 months). At 120 days of age, gonads were dissected for microscopic observation. Deviations of male ratios of hormone-treated fish from control were analyzed by Chi-square test. The populations of fish which the average of 62.88, 65.48 and 62.37% males in 125, 250 and 500 μgL^{-1} MT-treated groups, and 67.90, 84.94 and 75.12% males in 125, 250 and 500 μgL^{-1} mesterolone-treated groups, respectively were obtained. Control fish had an average male ratio of 58%, and this percentage was used as an expected ratio for Chi-square test. Immersion in MT at 250 μgL^{-1} resulted in significantly skewed sex ratio towards male ($P < 0.05$). Male ratios of fish obtained from mesterolone immersions at all concentrations were also significantly different from a male ratio of the control treatment ($P < 0.01$). Of the concentrations used in the present study, we were unable to produce all-male progeny. However, immersion with mesterolone at 250 μgL^{-1} was the most effective concentration that significantly skewed sex ratio, resulting in 27% male higher than that of untreated fish. Based on the results of this study, we therefore suggested that hormonal immersion using mesterolone at 250 μgL^{-1} was an applicable method for sex reversal in flowerhorn fish.

Key Words: Flowerhorn; Sex reversal; Mesterolone; 17 α-Methyltestosterone

Introduction

Flowerhorn is a crossbred fish which does not occur in the natural habitat. It was first bred in Malaysia between *Cichlasoma trimaculatum* and other cichlids species. These fish varieties were imported to Thailand as ornamental fish and became popular among aquarium keepers due to its natural beauty and its ability to grow in a wide range of environmental condition. Because of its worldwide popularity, this fish has been cultured and genetically bred continually to obtain new characteristics of customer's demand. In Thailand, male flowerhorn is commercially cultured extensively in the western region, particularly in Ratchaburi Province and sold both domestically and internationally. Like some other ornamental fishes, flowerhorn males are more attractive to customers than females because of their vibrant colors. Therefore, development of technique for all-male fish production would benefit fish farmers who produce flowerhorn. From the commercial point of view, hormonal sex reversal is the technique that is more convenient and less expensive compared to other methods, thus it is thought to be sensible for commercialization.

The most common steroid hormones that are used extensively in fish sex reversal include 17 β-estradiol and 17 α-methyltestosterone (Devlin and Nagahama, 2002). Hormonal sex reversal technique has been successfully implemented in many fish species especially in Tilapia (Gale et al., 1999; Pandian and Kirankumar, 2003; Wassermann and Afonso, 2003). Among androgens used for masculinization, 17 α-methyltestosterone is the most common hormone that has long been utilized for producing all-male progeny in many species of fish. In contrast, mesterolone, the less expensive androgen, is not widely applied for this purpose. There are only a few reports on the study of mesterolone on sex reversal in fish. In a study reported by Petchjul (2005), 94.6% male red tilapia was obtained by feeding 4-day-old fry with diet containing 60 mg mesterolone·Kg^{-1} feed for 21 days. However, in flowerhorn, sex reversal by means of hormonal administration has not yet been investigated. We thus attempted the application of these androgens for masculinization in this fish by hormonal immersion.

The main objectives of this study were (1) to determine the optimum concentration of hormone for fry immersion and (2) to assess the effects of hormone on growth and survival of hormone-immersed flowerhorn.

Materials and Methods

Broodstock rearing and breeding

One pair of mature male and female flowerhorn (body length of 12-15 cm) was raised in an aquarium (40x60x45 cm) containing 100 L of de-chlorinated freshwater. These male and female were partitioned by plastic net to prevent fighting. A 15 cm diameter-clay tray was placed in the female section as a egg-laying substrate. Fish were fed with fresh food (shrimp) or pellet feed *ad libitum* twice a day. Approximately 50-80% of water was changed twice a week. Plastic net was removed when the female was ready for breeding. Twelve hours after female laid eggs, tray was removed and placed to hatching glass aquarium in which air was slightly pumped and water was constantly circulated. Egg tray was removed when all newly hatched fry separated from substrate. First feeding fry (5-day-old) were fed with *Artemia* nauplii.

Hormone immersion and fish rearing

Twenty-one glass aquaria (40x60x45 cm dimension) were filled with 10 L of de-chlorinated freshwater. Each of 18 aquaria was added with 1 mL. of either 17 α-methyltestosterone or mesterolone solution (prepared by mixing hormone with 95% ethanol) to the concentrations of 125, 250 and 500 μgL^{-1}. Three control aquaria were added with 1 mL of ethanol. One hundred and forty 6-day-old fry (swim-up stage) were stocked in each aquarium. Twenty four hours after fry were exposed to the hormone, additional 10 L of water were added to each aquarium. Three days later, 10 L of water were

removed and additional water was added to the level of 80 L. From now on, regular water change (approximately 80%) was done twice a week.

Fry were fed twice a day with *Artemia* nauplii and 40% protein pellet for the subsequent period (90 days) until the fish reached 30 days old.

Growth measurement and sex determination

Once the experimental fish reached 4 months of age, individual body weight (g) and standard length (cm) of fish from each replicate were taken and fish gonads were dissected. Before dissection, fish were anesthetized for a few seconds in water containing clove oil. The abdomen was cut longitudinally and gonad was dissected out. Dissected gonad was then stained with Aceto-carmine, following the method as described by Wassermann and Afonso (2002) and observed under compound microscope (x40 and x100 magnification) for gonadal sex determination. Sex was judged as male or female by gonadal tissue showing oocyte or spermatogonial cells. Individual that oocyte or spermatogonia was not developed was recorded as undetermined sex.

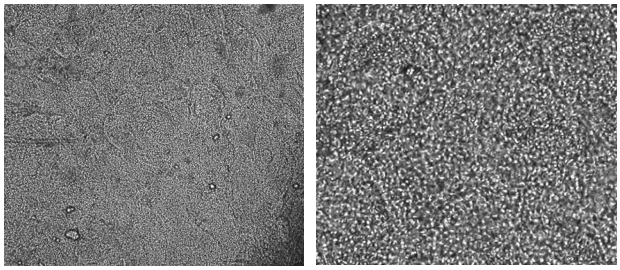
Data analysis

Percent male for each treatment was calculated by dividing the total number of males from all replicates with the total number of fish in that treatment. Chi-square analysis was used to test for the significance of the deviation from the expected male and female ratio of control fish. Statistical analyses of mean body weight, mean standard length and mean survival rate were performed using one-way analysis of variance and Duncan's multiple range test (DMRT). Body weight and standard length between male and female within each group were compared using T-test.

Results

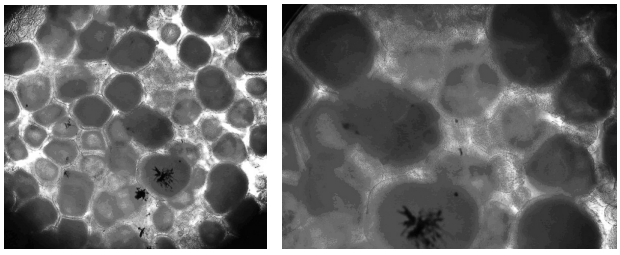
Histological characteristics of reproductive tissue

Histological examination of fish gonads revealed that male gonads composed of spermatogonia (Figure 1 A and B), whereas female gonads had oocytes (Figure 2 A and B).



(1A) X 40 (1B) X 100

Figure 1 Male gonad observed under compound microscope



(2A) X 40 (2B) X 100

Figure 2 Female gonad observed under compound microscope

Table 1 Experimental treatment

Treatment	Hormone Concentrations
1	Control
2	immersion in 17 α-Methyltestosterone at 125 μgL^{-1}
3	immersion in 17 α-Methyltestosterone at 250 μgL^{-1}
4	immersion in 17 α-Methyltestosterone at 500 μgL^{-1}
5	immersion in Mesterolone at 125 μgL^{-1}
6	immersion in Mesterolone at 250 μgL^{-1}
7	immersion in Mesterolone at 500 μgL^{-1}

Effect of hormones on sex ratio

At the termination of the experiment, the immersion of 6-day-old fry in 17 α-methyltestosterone and mesterolone resulted to the difference in sex ratios from the expected ratio of untreated fish which served as control (Table 2). While control fish had 58.04% male, fish that were immersed in 17 α-methyltestosterone at the concentration of 125, 250 and 500 μgL^{-1} had 62.88, 65.48 and 62.37% males, respectively. Immersion in mesterolone at the concentrations of 125, 250 and 500 μgL^{-1} resulted in 67.90, 84.94 and 75.12% males, respectively. The Chi-square test for difference in sex ratios between hormone-immersed groups and control revealed that

Table 2 Percentage of male flowerhorn at 120 days of age

Treatment	Total (individual)	Male (individual)	Female (individual)	Percentage of male (%)
Control	286	166	120	58.04
17 α-Methyltestosterone				
125 μgL^{-1}	264	166	98	62.88^{ns}
250 μgL^{-1}	252	165	87	65.48^{*}
500 μgL^{-1}	194	121	73	62.37^{ns}
Mesterolone				
125 μgL^{-1}	271	184	87	67.90^{**}
250 μgL^{-1}	259	220	39	84.94^{**}
500 μgL^{-1}	201	151	50	75.12^{**}

Remarks: 1) ns: non-significant difference ($P > 0.05$) 2) * : Significant difference ($P < 0.05$) 3) ** : Highly significant difference ($P < 0.01$). Sex ratio (male:female) of control group (0.58: 0.42) was used as an expected value for Chi-square test.

Table 3 Percent survival of 120-day-old juvenile flowerhorn (Mean±SD)

Treatment	Total (individual)	Male (individual)	Female (individual)	Unidentified sex (individual)	Survival rate (%)
Control	295	166	120	9	70.20 ± 8.90^{a}
17 α-Methyltestosterone					
125 μgL^{-1}	271	166	98	7	64.50 ± 8.02^{a}
250 μgL^{-1}	252	165	87	0	60.00 ± 10.40^{a}
500 μgL^{-1}	231	121	73	37	55.00 ± 10.00^{a}
Mesterolone					
125 μgL^{-1}	271	184	87	0	64.50 ± 17.80^{a}
250 μgL^{-1}	266	220	39	7	63.30 ± 15.90^{a}
500 μgL^{-1}	261	151	50	60	62.10 ± 15.10^{a}

Remarks: Means followed by the same letters are not significantly different ($P > 0.05$)

Table 4 Effect of hormone on growth of flowerhorn fish (Mean±SD)

Treatment	Average body weight (g)	Average standard length (cm)
Control	2.13 ± 0.42^{a}	4.55 ± 0.24^{a}
17 α-Methyltestosterone		
125 μgL^{-1}	3.93 ± 0.71^{b}	4.62 ± 0.27^{a}
250 μgL^{-1}	4.27 ± 0.47^{b}	4.76 ± 0.20^{a}
500 μgL^{-1}	4.27 ± 0.07^{b}	4.82 ± 0.19^{a}
Mesterolone		
125 μgL^{-1}	3.98 ± 1.20^{b}	4.67 ± 0.47^{a}
250 μgL^{-1}	2.62 ± 0.20^{a}	4.74 ± 0.04^{a}
500 μgL^{-1}	2.25 ± 0.42^{a}	4.54 ± 0.26^{a}

Remarks: For each column, means followed by different letters are significantly different by DMRT ($P < 0.05$)

Table 5 Comparisons of body weight and standard length between male and female within treatment.

Treatment	Weight (g)			Standard length (cm)		
	Male	Female	(T-test)	Male	Female	(T-test)
Control	3.8	3.5	1.111^{ns}	4.6	4.5	1.505^{ns}
(n=286)	(n=166)	(n=120)		(n=166)	(n=120)	
17 α-Methyltestosterone						
125 μgL^{-1}	4.0	3.8	0.740^{ns}	4.7	4.5	1.013^{ns}
(n=264)	(n=166)	(n=98)		(n=166)	(n=98)	
250 μgL^{-1}	4.2	4.2	0.295^{ns}	4.8	4.7	0.389^{ns}
(n=252)	(n=165)	(n=87)		(n=165)	(n=87)	
500 μgL^{-1}	4.4	4.1	0.665^{ns}	4.9	4.7	1.370^{ns}
(n=194)	(n=121)	(n=73)		(n=121)	(n=73)	
Mesterolone						
125 μgL^{-1}	3.9	3.5	1.375^{ns}	4.6	4.5	0.820^{ns}
(n=271)	(n=184)	(n=87)		(n=184)	(n=87)	
250 μgL^{-1}	4.2	4.1	0.222^{ns}	4.8	4.6	0.671^{ns}
(n=259)	(n=220)	(n=39)		(n=220)	(n=39)	
500 μgL^{-1}	4.0	3.8	2.161^{*}	4.7	4.2	2.816^{**}
(n=201)	(n=151)	(n=50)		(n=151)	(n=151)	

Remarks: 1) ns: Non significant difference ($P > 0.05$) 2)* : Significant difference ($P < 0.05$) 3)** : Highly significant difference ($P < 0.01$)

male ratios of fish immersed in mesterolone at 125, 250 and 500 μgL^{-1} were significantly higher ($P < 0.01$) than that of control. Among the treatments receiving 17 α-methyltestosterone, only fish that were immersed a 250 μgL^{-1} had higher male ratio than control ($P < 0.05$).

Effect of hormones on survival

Survival rates of fish at the end of experiment (120 days of age) among treatments were not statistically significant different (Table 3).

Effect of hormones on growth

Flowerhorn fishes immersed in 17 α-methyltestosterone at the concentrations of 125, 250 and 500 μgL^{-1} and mesterolone at the concentrations of 125 μgL^{-1} had average body weights higher than those of other treatments (Table 4). Hormone treatment had no effect on the length of this fish (Table 4), in which the average standard lengths ranged between 4.54 - 4.82 cm.

For growth comparison between sexes, fish body weight and standard length of male and female within each treatment were more or less similar (Table 5). However, the group immersed in 500 μgL^{-1} mesterolone had male which had higher growth rate than female in both body weight and length.

Discussion

Histological study on the gonad of the flowerhorn fishes at 120 days old by immersion method showed that sex of the this fish can be differentiated by observing oocytes and spermatogonia with a compound microscope. Wassermann and Afonso (2002) studied a sex differentiation of tilapia and reported that sex determination in tilapia fish was more reliable when the fish sample was 45-47 days old after hatching with the weight of more than 0.5 g and the length of 3.2 cm. In this study, however, we found that the sex differentiation of 60-day-old flowerhorn had not yet well developed and was difficult to determine their sexes.

Immersion of fishes in the solution of 17 α-methyltestosterone, showed a slight increase of the male sex in the hormone treatment compared with the untreated fish. Gale et al. (1999) also reported that immersing tilapia fish (10 and 13 days after hatching) in 500 μgL^{-1} 17 α-methyltestosterone for 3 hours did not alter the sex of this fish. This may be due to the fact that the 17 α-methyltestosterone had been chemically changed into the form which possessed lower efficacy and this hormone may be excreted very rapidly from the fish's body.

Immersed tilapia once after 14 days post hatching or twice after 10 and 14 days post hatching in 1,800 μgL^{-1} the of 17 α-methyltestosterone for 4 hours proved to be successful to alter the sex of tilapia, with the ratio of 91% male sex for single immersion and 98% male sex for double immersion (Wassermann and Afonso, 2003).

In this study, flowerhorn fish had lower percentage of male when the concentration of the 17 α-methyltestosterone had increased (at 125, 250 and 500 μgL^{-1}) respectively, when compared with the non treated control. For the treatment with mesterolone, the result showed that male sex ratio of flowerhorn fish was statistically different from the non treated control. At 250 μgL^{-1}, the male sex ratio was of 84.94 %, higher than that of the control about 27 %. At 500 μgL^{-1}, the male sex ratio was of 75.12 %. This showed that mesterolone was more effective than 17 α-methyltestosterone in altering the sex of this fish. Mesterolone, as a derivative of dihydrotestosterone (DHT), could not be chemically transformed to estrogen and it was believed that DHT was 30 times more effective than testosterone (Hemat, 2004)

Conclusion

In this study, an attempt was made to develop method for sex reversal by hormone immersion using two androgens: 17 α-methyltestosterone (MT) and mesterolone. Flowerhorn fish immersed in 125, 250 and 500 μgL^{-1} MT had 62.88 %, 65.48 % and 62.37 % male, respectively while those immersed in 125, 250 and 500 μgL^{-1} mesterolone had 67.90 %, 84.94 % and 75.12 % male, respectively. The male ratio of

untreated group was 58.04 %. The 250 μgL^{-1} MT-treated group and all mesterolone-treated groups had significantly higher male ratio than untreated fish at $P < 0.05$ and $P < 0.01$, respectively. In terms of the effects of hormone on growth and survival, fish immersed in hormone at the studied levels showed no significant difference in growth and survival rates. However, flowerhorn fish immersed in MT and mesterolone had the declining survival rate with an increasing hormone concentration. Although there was no experimental group producing all-males, the result showed that immersed fish in 250 μgL^{-1} of mesterolone would yield 85 % male sex ratio.

Acknowledgements

The authors would like to thank Assoc. Prof. Dr. Thanit Pewnim, at the Faculty of Sciences, Silpakorn University, Nakhon Pathom campus, Thailand, for his research guidance. Mr. Frank Werth, the English teacher at the Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Phetchaburi IT campus, Phetchaburi, Cha-Am, Thailand, is appreciated for his editing work for proof reading the usage of written English. This research was financially supported in part by Silpakorn University Research and Development Institute, Thailand.

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