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Effect of Norethindrone Acetate on Masculinization, Growth and Reproduction in Zebrafish *Danio rerio*

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Abstract

To masculinize *Danio rerio*, Norethindrone acetate (NE) was administered through discrete immersion (2 hrs each) from the 18th to the 25th day after hatching (dph) at selected concentrations (100, 200, 400, 800, 1,000 and 1,200 $\mu g \cdot l^{-1}$). The immersion at a concentration of 1,000 $\mu g \cdot l^{-1}$ ensured 100 % masculinization and the highest survival (97 %) after treatment and at the age of puberty. Barring this concentration group, the NE acted as a growth suppressant and the magnitude of its suppression was stronger in females. During the 240 day experiment, sexual maturity in females was postponed from 120th dph in the control to 270th dph in those treated at 1,200 $\mu g \cdot l^{-1}$, and reduced spawning frequency (15 - 3 times) and cumulative fecundity (1,168 - 111 eggs) by reducing the number of vitellogenic eggs. However, in the treated males, sperm motility was reduced (105 – 73 sec); consequently, fertilizability of the milt drawn from these males was also reduced from 91 % to 78 %. Progeny testing showed that the cross between males treated at >800 $\mu g \cdot l^{-1}$ and normal females generated the presumed 'homogametic' males, which however, could induce the females to spawn fewer eggs than that of the presumed 'heterogametic' males.

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Introduction

In aquaculture, hormonal treatment is a simple method to generate monosex progenies. Of the 16 tested androgens, 17α-methyltestosterone (MT) is the most widely used steroid to generate all male progenies in the ornamental fish like the fighter fish *Betta splendens*, in which males are more attractive than females, and food-fish, like the Mossambique tilapia *Oreochromis mossambicus*, wherein rearing of all male progenies avoids frequent spawning and consequent stunted growth (Pandian and Sheela 1995). However, discrete immersion of the fighter fish fry to 900 µg MT•l^{-1} for a brief period of 3 hrs, despite masculinizing the fighter fish, reduced body growth of the male to less than 50 % of the control; when allowed to court, the stunted male was not readily acceptable to a normal female and when accepted, it could induce its partner to spawn less than 50% eggs only (Kirankumar and Pandian 2002). Varadaraj (1990) showed that oral administration of the rarely used norethindrone acetate (NE) produced 100% male tilapia, which weighed 40% more than the control. His publication established for the first time that NE is not only a potent androgen but also a powerful anabolic steroid. However, it is recently shown that in the fighter fish fry, subjected to discrete immersion for a period of 9 hrs at doses ranging from 250 to 1250 µg•l^{-1}, NE acted as an anabolic steroid in the males but as a mild catabolic one in the treated but persisting females (Balasubramani 2006). Hence, it is chosen to generate all male progenies in the zebrafish (*Danio rerio*) to know whether NE is an anabolic steroid in both sexes.

Materials and Methods

Experimental fish

To study the effect of NE, the zebrafish, *Danio rerio* Hamilton-Buchanan 1822, a cyprinid experimental model (Petersen et al. 2001) was chosen. From S. Ram Fish Farm, Madurai, healthy adults were purchased in the spring of 2003 and reared in four large circular aquaria (each with 150 cm diameter x 180 cm height) containing well aerated water. From these healthy brooders, hatchlings were obtained for experiments. The hatchlings depend on yolk; following the 2nd or 3rd dph, they were fed paramecium and boiled yolk granules of hen's egg, subsequently, *Artemia* nauplii for a week and synthetic pellet food from the 20-25th dph. The feed ensured a supply of 30% protein (David and Pandian 2006). The adult fish (brood stock) were fed *Chironomus* larvae and/or *Tubifex tubifex* ad *libitum* twice a day.

Following prolonged observations, potential breeders were identified 12 hr prior to spawning. The identified male and female were released into a breeding aquarium (10 litre) filled with water. Spawning occurred the next day between 5 and 7 a.m.; however, for experimental purpose, the identified individuals were allowed to almost complete courtship until 5 am and the gametes were stripped for the experiment.

Norethindrone acetate (NE)

The NE (Sigma, St. Louis, MO) was chosen to masculinize the zebrafish by discretely immersing the fry for a cumulative period of 14 hrs in water containing selected concentration of NE. Stock solution was prepared in absolute alcohol at a concentration of 1 mg•ml^{-1}. Aliquot of stock solution was added appropriately to a glass bowl containing one litre of water to get the final steroid concentration for each treatment group accordingly. Hormonal treatment was carried out by transferring the fry into a glass bowl containing water with dissolved steroid. Seven groups of 30 fry each were subjected to discrete immersion at NE concentrations of 0, 100, 200, 400, 800, 1000 and 1200 µg•l^{-1} water. There were 5 replicates (batches) for each treatment group.

Discrete immersion

Owing to its being cost-effective, allows limited handling, and reduces pollution; the discrete immersion technique was adopted. The fries were immersed for 2 hrs each on the 18th, 19th, 20th, 22nd, 23rd, 24th and 25th days post hatching (dph) (Uchida et al. 2002). When treatments on the selected days were completed, the experimental series was transferred to rearing aquaria and the procedure was repeated. Upon completion of the entire sequence of immersion, the surviving fry in each batch were counted and released into a rearing aquarium (45 L x 30 B x 15 H cm) containing water (approximately 26° C; 5.5 mg O_2•l^{-1}); 14 L:10 D and were fed *ad libitum*. Subsequently, monthly growth measurement (total length) was made; for this randomly selected 10 individuals in each batch were anesthetized and kept in a glass tray, at the bottom of which a transparent graph (mm) sheet was pasted. The measurements were made keeping the tray under a stereomicroscope (10 X, Nikon, Japan).

Sex ratio

From the 90th dph onwards, a male was easily recognized through its slender body shape and a female from its relatively larger size and bigger belly. From the 120th dph onwards, stripping with the resultant milt or eggs readily confirmed the sex of the individual.

Records were maintained for each experimental batch the age at which puberty (sexual maturity) was attained, and the frequency of spawning and milting as a function of age for a period of 240 days.

Motility

On a microscopic slide with a well, 300 µl of tap water was added to 100 µl Ringer's saline containing milt, and sperm motility duration was estimated using a stopwatch (Shinco, India). The duration of motility was taken in seconds, when 50 % of the sperm cells stopped motility (Billard and Cosson 1992).

Fecundity

Upon attaining sexual maturity, the persisting female was paired with a normal male and the eggs released during successive spawnings were separately collected and counted.

Fertilizability

An individual male was introduced to mate the selected female and fertilizability (%) was calculated by counting the number of dividing eggs as percentage of the total number of eggs.

Hatchability

The collected eggs were thoroughly rinsed in clean water before being transferred into a glass bowl (100 ml). A glass pipette, with an opening diameter of approximately 2 mm, was used to handle the eggs and fry. After 24 hr, unfertilized and arrested embryos were removed from the bowl and the developing eggs were observed until hatching. Hatchability was calculated by counting the number of hatchlings as percentage of the number of eggs.

Histology

After sexual maturity, three treated individuals from each series were anesthetized using clove oil in tap water (2 ml•l^{-1}). One of their gonads was dissected out, fixed in 10 % formalin and subsequently mounted in the tissue freezing medium (Junk, Leica Instruments GmbH, Germany) at -17° C to obtain 6 μm thin sections in the freezing microtome (Minotome-Microtome Cryostat; I.E.C., Needham Heights, MA, USA).

These sections on the slide were fixed in methanol (100 %) and kept overnight. Subsequently, the slide was rinsed in tap water followed by distilled water for 1 min and stained with haematoxylin and eosin, following the standard procedure described in the manual catalogue card number 74-33828 of International Equipment Company, MA, USA. Then the sections were permanently mounted on the slide with DPX. They were scanned and photographed using a phase contrast microscope (Nikon Optiphot, Nikon Corporation, Tokyo, Japan).

Progeny testing was carried out by randomly selecting 2-5 males in each of the 3 groups. The selected males from the first, second and third groups belonged to those treated at the concentrations < 1,000 μg•l^{-1} (GI 100, GI 200, GI 400, GI 800; designated as 'sub-optimal', (Table 5), those at 1,000 μg•l^{-1} (GII, designated as 'optimal') and those at 1,200 μg•l^{-1} (GIII, 'super-optimal'), respectively.

Statistical analysis

Data analyses were based on their mean ± SD. The chi-square test (χ^2) was used to test the null hypothesis of no difference of male:female sex ratio between control and treated groups. Multiple comparison tests were performed for survival of fry, milting period, sperm motility,

fertilizability, spawning period, fecundity and hatchability between control and treated series after One-way ANOVA. All the statistical analyses were performed using Sigmastat ver. 2.0.

Results

Survival

Following the cumulative immersion period of 14 hrs from the 18th to 25th dph at all tested NE concentrations, 3 to 11% mortality occurred (Table 1). Also at puberty, the zebrafish suffered mortality of 3-17%. The immersion at 1,000 µg•l^{-1} resulted in not only 100% masculinization but also ensured the highest survival of 97% both at the end of the treatment and on the day of sexual maturity. At any tested treatment dose, no deformed fry, live or dead, was found.

Table 1. Effect of different doses of norethindrone acetate on survival and sex reversal of the zebrafish *Danio rerio*, which were previously immersed for 14 hours, i.e. 2 hours each on the 18th, 19th, 20th, 22nd, 23rd, 24th and 25th day after hatching. Each value represents the mean performance of 5 batches each consisting of 30 fry

Group	Dose (µg/l)	Survival at the				Sex distribution (no)		Sex ratio[2] ♂:♀
		end of treatment[1]		sexual dimorphism[1]				
		(no)	(%)	(no)	(%)	♂	♀	
Control	0	29 ± 0.9	98 ± 3.0 [a]	28 ± 0.8	93 ± 2.8 [c]	14 ± 0.8	14 ± 0.8	0.50:0.50
(G I)	100	27 ± 0.7	90 ± 2.4 [a]	25 ± 0.8	83 ± 2.8 [d]	19 ± 0.5	6 ± 0.7	0.76:0.24*
(G I)	200	28 ± 0.8	93 ± 2.8 [a]	27 ± 0.5	89 ± 1.8 [c]	22 ± 1.3	5 ± 1.5	0.81:0.19*
(G I)	400	27 ± 0.8	89 ± 2.8 [b]	26 ± 0.7	87 ± 2.4 [c]	24 ± 0.8	2 ± 0.5	0.92 :0.08*
(G I)	800	27 ± 0.7	90 ± 2.4 [b]	26 ± 1.3	87 ± 4.3 [c]	23 ± 1.3	3 ± 0.5	0.92 :0.08*
(G II)	1000	29 ± 0.8	97 ± 2.8 [a]	29 ± 0.8	97 ± 2.8 [c]	29 ± 0.8	0	1.00:0.00*
(G III)	1200	28 ± 0.6	92 ± 1.8 [b]	26 ± 1.5	86 ± 4.9 [d]	24 ± 2.2	2 + 0.7	0.92 :0.08*

[1] All values are mean ± SD; 'b' and 'd' are significantly ($p<0.001$) different from the respective control values namely 'a' and 'c', (Multiple comparison Dunnett's method after One Way ANOVA)

[2] Chi square test with Yates correction * indicates significant deviation ($p<0.001$) from the expected 0.5:0.5 ratio of males to females

Growth

On the day of hatching, the fry measured a body length of 3.3 mm in the control and all the groups in the treated series. The log phase of accelerated growth was sustained until the 90th dph in both series and the observed differences in body length between the control and treated series were significant (Table 2). However, from the 120th dph, the control female grew faster than the male and attained a longer body length of 38 mm on the 240th dph, while the control male attained the length of 33 mm only.

In all the treated groups, the females began to suffer growth suppression from the 120th dph, when sexual dimorphism became apparent and suppression became increasingly more and more apparent, as the days advanced to 240th dph. The group at 1,200 µg•l^{-1} suffered relatively stronger suppression than those at concentrations below 1,000 µg•l^{-1}. However, at the concentration of 1,000 µg•l^{-1}, the males grew almost as fast as the control and attained a body length of 32 mm on the 240th day, which was not significantly less than that (33 mm) of the control male.

Table 2. Effect of different doses of NE on the body length (mm) of the zebrafish, whose fry were previously immersed for a cumulative period of 14 hours, i.e. 2 hours each on the 18th, 19th, 20th, 22nd, 23rd, 24th and 25th day after hatching. Each value represents the mean of growth of randomly selected 10 fry each from 5 batches.

Dose (µg/l)	**1st day**	**60th day**	**90th day**	**120th day**		**240th day**	
				♂	♀*	♂	♀*
Control	3.3 ± 0.3	11 ± 0.8 [a]	18 ± 0.8 [c]	21 ± 1.4 [e]	25 ± 1.4 [g]	33 ± 2.3 [i]	38 ± 2.0 [k]
100	3.3 ± 0.3	10 ± 0.7 [b]	18 ± 0.4 [c]	20 ± 0.5 [e]	22 ± 1.3 [h]	30 ± 2.2 [j]	33 ± 2.1 [l]
200	3.3 ± 0.3	11 ± 0.4 [b]	18 ± 0.3 [c]	20 ± 0.6 [f]	23 ± 1.2 [h]	31 ± 1.8 [j]	33 ± 2.0 [l]
400	3.3 ± 0.3	11 ± 0.4 [b]	19 ± 0.3 [d]	21 ± 0.4 [e]	24 ± 0.8 [g]	31 ± 1.9 [j]	33 ± 2.3 [l]
800	3.3 ± 0.3	11 ± 0.4 [b]	19 ± 0.3 [d]	21 ± 0.3 [e]	24 ± 0.8 [g]	31 ± 1.5 [j]	34 ± 1.7 [l]
1000	3.3 ± 0.3	11 ± 0.4 [b]	19 ± 0.5 [d]	22 ± 0.3 [f]	-	32 ± 1.9 [j]	-
1200	3.3 ± 0.3	11 ± 0.3 [b]	18 ± 0.3 [d]	21 ± 0.8 [e]	24 ± 1.0 [g]	29 ± 1.7 [j]	32 ± 2.0 [l]

All values are mean ± SD; 'b', 'd', 'f', 'h', 'j', and 'l' are significantly ($p<0.001$) different from their respective control values namely 'a', 'c', 'e' 'g', 'i' and 'k' (Multiple comparison *Tukey test or with Dunnett's method after One Way ANOVA)

Sex ratio

With increasing concentration of NE, the sex ratio was progressively biased towards male (Table 1). The treatment dose of 1,000 µg•l^{-1} resulted in the production of 100% males. However, upon increasing the dose to 1,200 µg•l^{-1}, there was a paradoxical sex reversal of 8% among females.

Female

The NE dose-dependently and significantly extended the age of first spawning and inter-spawning periods, and reduced the fecundity and hatching of F1 fry. Table 3 also summarizes the cumulative effects of the NE on the frequency of spawning and fecundity of F_0 females and production of F_1 fry during the experimental period of 240 days. The spawning frequency and cumulative hatchability of F_1 fry were significantly reduced.

Table 3. Effect of different doses of NE on the number of spawning and cumulative fecundity of the treated zebrafish F_0 females during 240 day experiment. The F_0 treated females were crossed with normal males. Each value represents the mean of 5 estimates.

Group	Dose (µg/l)	Spawning frequency (no)	Fecundity (no)	Hatchlings (no)
Control	0	15 ± 0.8^{a}	1283 ± 67^{c}	1168 ± 61^{e}
(G I)	100	9 ± 1.3^{b}	725 ± 72^{d}	558 ± 55^{f}
(G I)	200	8 ± 1.0^{b}	581 ± 28^{d}	482 ± 24^{f}
(G I)	400	7 ± 0.5^{b}	499 ± 48^{d}	394 ± 38^{f}
(G I)	800	5 ± 0.5^{b}	264 ± 17^{d}	163 ± 10^{f}
(G III)	1200	3 ± 0.5^{b}	196 ± 10^{d}	111 ± 6^{f}

All values are mean ± SD; 'b', 'd' and 'f' are significantly ($p<0.001$) different from their respective control values namely 'a', 'c' and 'e' (Multiple comparison Tukey test after One Way ANOVA)

Figure 1 shows the degenerative changes undergone by the oocytes and related ovarian structures of the treated females. The following changes were recognized: i) progressive reduction in the number of pre-vitellogenic oocytes, ii) progressive irregularization of the shape of oocytes and iii) progressive increase in the number of enlarged vacuoles and the degraded products. However, vitellogenesis, though apparently slowed down, was not totally inhibited, as vitellogenic oocytes were visible in the ovary of the female treated at 1,200 µg NE•l^{-1}. Consequently, the generation of critical minimum number of, say 60 eggs, matured oocytes 'ready for spawning' required longer intervals between the successive spawnings, when the zebrafish were exposed to higher concentrations of NE.

Male

On the 120th dph, the control males attained sexual maturity and readily yielded milt, when stripped. Irrespective of the dose to which the males were exposed, they attained sexual maturity on the 121st dph (Table 4). However, the inter-milting period, was prolonged to 5 - 6 days in the treated series. Consequent to the decrease in sperm motility, fertilizability of sperm, when allowed to fertilize eggs spawned by the normal females, decreased from 91% to 78%.

Table 4. Effect of different doses NE on the age of maturity, inter-milting period and fertilizability of sperm drawn from the treated and sex reversed male zebrafish which were previously subjected to discrete immersion for 14 hours, i.e. 2 hours each on the 18th, 19th, 20th, 22nd, 23rd, 24th and 25th day after hatching. These sex reversed males were crossed with normal females. Each value represents the mean of 5 estimates.

Dose (µg/l)	Age of maturity (dph)	Inter-milting period (day)	Sperm count (no x 10^6/ml)	Motility duration (sec)	Fertilizability (%)
Control	120 ± 1.4 ^{a}	4 ± 0.4 ^{b}	4.9 ± 0.43 ^{d}	105 ± 7.9 ^{f}	91 ± 2.7 ^{h}
100	121 ± 1.1 ^{a}	6 ± 0.5^c	4.2 ± 0.5 ^{e}	73 ± 2.9 ^{g}	78 ± 2.7 ^{i}
200	121 ± 2.3 ^{a}	5 ± 0.6 ^{b}	4.1 ± 0.3 ^{e}	85 ± 3.6 ^{g}	82 ± 5.7 ^{i}
400	121 ± 2.3 ^{a}	5 ± 0.8 ^{b}	3.6 ± 0.2 ^{e}	84 ± 3.5 ^{g}	81 ± 4.2 ^{i}
800	121 ± 2.3 ^{a}	5 ± 0.5 ^{b}	3.8 ± 0.3 ^{e}	88 ± 2.5 ^{g}	88 ± 3.2 ^{h}
1000	120 ± 1.7 ^{a}	5 ± 0.8 ^{b}	4.1 ± 0.2 ^{e}	88 ± 2.5 ^{g}	89 ± 4.2 ^{h}
1200	121 ± 1.8 ^{a}	6 ± 0.8 ^{b}	3.7 ± 0.3 ^{e}	73 ± 3.4 ^{g}	78 ± 2.9 ^{i}

All values are mean ± SD; 'c', 'e', 'g' and 'i' are significantly ($p<0.05$) different from their respective control values namely 'b', 'd', 'f' and 'h' (Multiple comparison Tukey test after One Way ANOVA)

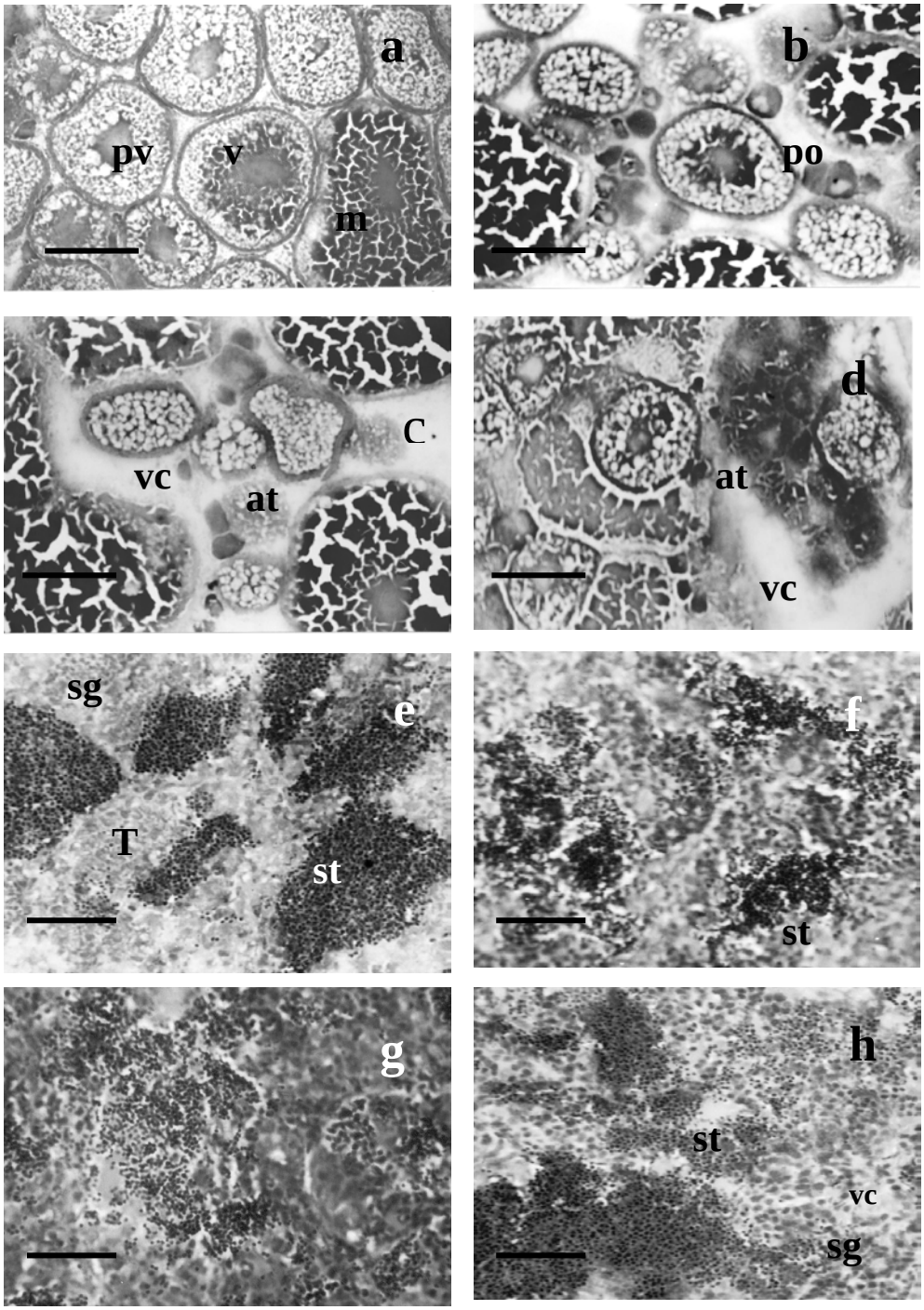

Fig. 1 Effect of different doses of NE on the ovary (a-d) and testis (e-h) of zebrafish *Danio rerio* (a) control; (b) 100 µg/l; (c) 800 µg/l; (d) 1200 µg/l. po – primary oocyte, pv - pre-vitellogenic oocyte; v - vitellogenic oocyte; vc - vacuole; m – matured oocyte; dp - degraded product; at – atretic oocyte; Scale ▬▬ 300 µm; (e) control; (f) 400 µg/l; (g) 800 µg/l; (h) 1000 µg/l; sg – spermatogonium; st – spermatids, T – seminiferous tubule; vc – vacuole. Scale ▬▬ 25 µm.

Histology

The causes for the observed reduction in the number of sperm cells are the following: i) the most obvious reduction or the disappearance of the spermatogonia (Fig. 1f), ii) the enlargement and the consequent loss of intactness (Fig. 1h) of the seminiferous tubules and iii) the remarkable decrease in the density of spermatids.

Progeny testing

In zebrafish, Traut and Winking (2001) failed to identify the sex chromosomes, hence the genetic mechanism for sex determination of the zebrafish is yet to be known. For an easier understanding, it is chosen for the present to designate the phenotypic female and male as the presumed 'homogametic' (X^1X^2) and presumed 'heterogametic' (X^1Y^2), respectively.

Of the 15 randomly selected males, 5 from each of the groups G I, G II and G III were subjected to progeny testing; of them, 9 sired F_1 progenies, whose sex ratio was about 0.5♂:0.5 ♀ (Table 5). But the sex ratio of the progenies sired by G I 4, 800, G I 5 800, G II 2, G II 3, G II 5 and G III 3 were more than 0.75♂ : 0.25 ♀. Hence, the genotype of these male parents is presumed to be 'homogametic'. Clearly, the group exposed to concentrations less than 800 µg NE•l^{-1} could not generate the presumed 'homogametic' male and/or ensure its survival. At a dose of 1,000 µg NE•l^{-1}, the 60% males were 'homogametic'. At a concentration of 1,200 µg NE•l^{-1}, only 20% males were presumably 'homogametic'. Hence, the treatment at 800, 1,000 and 1,200 µg•l^{-1} ensured not only a higher percentage of masculinization (more than 92%) but also the survival of the presumed 'homogametic' males. Therefore, for the development of brood stock of 'homogametic' males, the treatment of 1,000 µg•l^{-1} of norethindrone acetate is recommended.

With the identification of the presumed genotype of the treated males and the number of progenies that they were able to generate from the eggs released in a single spawn by a normal female, it was possible to estimate the number of progenies produced by the females paired with the treated 'homogametic' or 'heterogametic' males. Table 6 shows that irrespective of the presumed genotype borne by the males, the NE-treatment at all the tested concentrations significantly reduced the number of progenies produced by the normal females, when paired with these males. Since only normal females were used to pair with the treated males, each female is expected to spawn 89 eggs, from which 83 progenies hatched out. However, the ability of all the treated males to induce the females to release eggs was not equal to that of a control male, and this resulted in the production of 63 - 72 progenies alone. Notably, the difference between the number of progenies generated by the presumed 'heterogametic' and that by the presumed 'homogametic' male is not significant. In other words, both the presumed 'heterogametic' and the sex reversed 'homogametic' males induced the females to spawn equal number of eggs.

Table 5. Progeny testing of randomly selected male zebrafish treated at sub-optimal (G I), optimal (G II) and super-optimal (G III) doses of NE. The number following the first column in G I indicates the sub-optimal doses of 100, 200, 400 and 800 µg/l. Each selected sire was allowed to pair with 2 or 3 randomly selected normal females.

Sire (no)	Dam (no)	Progeny (no)	Progeny sexed (no)	Sex distribution (no)		Sex ratio ♀:♂	Presumed genotype of ♂ parent
				♀	♂		
Control	2	80	40	21	19	0.53:0.47	X^1Y^2
	5	85	40	20	20	0.50:0.50	
G I 1 100	3	68	35	18	17	0.51:0.49	X^1Y^2
	4	70	35	18	17	0.51:0.49	
G I 2 200	5	66	30	16	14	0.53:0.47	X^1Y^2
	6	74	30	15	15	0.50:0.50	
G I 3 400	4	74	30	16	14	0.53:0.47	X^1Y^2
	3	68	33	17	16	0.52:0.48	
G I 4 800	4	62	35	26	9	0.74:0.26	X^1X^2
	7	58	35	25	10	0.71:0.29	
	6	64	40	30	10	0.75:0.25	
G I 5 800	7	68	32	25	7	0.78:0.22	X^1X^2
	2	62	35	27	8	0.77:0.23	
	8	58	30	23	7	0.77:0.23	
G II 1	7	65	32	17	15	0.53:0.47	X^1Y^2
	4	76	35	17	18	0.49:0.51	
G II 2	5	64	40	29	11	0.73:0.27	X^1X^2
	6	70	35	26	9	0.74:0.26	
	9	62	40	31	9	0.78:0.22	
G II 3	5	68	40	32	8	0.80:0.20	X^1X^2
	3	70	40	31	9	0.78:0.22	
	2	64	40	30	10	0.75:0.25	
G II 4	6	76	35	18	17	0.51:0.49	X^1Y^2
	4	70	35	17	18	0.49:0.51	
G II 5	8	63	30	23	7	0.77:0.23	X^1X^2
	3	59	30	24	6	0.80:0.20	
	7	57	30	24	6	0.80:0.20	
G III 1	5	76	40	21	19	0.53:0.47	X^1Y^2
	2	82	40	22	18	0.55:0.45	
G III 2	4	78	40	22	18	0.55:0.45	X^1Y^2
	3	84	40	21	19	0.53:0.47	
G III 3	5	70	35	26	9	0.74:0.26	X^1X^2
	6	66	35	25	10	0.71:0.29	
	3	56	35	26	9	0.74:0.26	
G III 4	1	68	35	18	17	0.51:0.49	X^1Y^2
	8	62	30	16	14	0.53:0.47	
G III 5	7	48	30	16	14	0.53:0.47	X^1Y^2
	2	64	30	16	14	0.53:0.47	

Table 6. Effect of different doses of NE on progeny producing ability of the male zebrafish bearing different genotypes. Each genotype identified male was allowed to cross 2 – 3 normal female. Each value represent the mean of 2 – 3 males for each of the Groups I, II and III

Group	Genotype and sex	Progeny produced (no)
Control	X^1Y^2 ♂	83 ± 3 [a]
Group I*	X^1Y^2 ♂	69 ± 4 [b]
	X^1X^2 ♂	63 ± 4 [b]
Group II*	X^1Y^2 ♂	72 ± 4 [a]
	X^1X^2 ♂	64 ± 4 [b]
Group III*	X^1Y^2 ♂	70 ± 6 [a]
	X^1X^2 ♂	64 ± 7 [b]

All values are mean SD; 'b' is significantly ($p<0.05$) different from the respective control 'a' (Multiple comparison Tukey test after One Way ANOVA)

* Student's t-test was performed to compare the difference in the progeny production between G I X^1Y^2 ♂ and X^1X^2 ♂ ($t = 1.961$, $p = 0.121$); G II X^1Y^2 ♂ and X^1X^2 ♂ ($t = 2.460$, $p = 0.070$); G III X^1Y^2 ♂ and X^1X^2 ♂ and found that the differences were not significant ($t = 1.143$; $p = 0.317$).

Discussion

Since publications available on the effects of NE on masculinization are few (googlescholar and PubMed searched with key words: norethindrone-masculinization-teleost fish), this discussion is limited. At a concentration of 1,000 µg•l^{-1}, the NE not only ensured 100 % masculinization but also the highest survival (97%) following the treatment as well as at the age of puberty. At the same concentration of 1,000 µg•l^{-1}, it could, however, ensure a maximum of 92% masculinization in the fighter fish *Betta splendens* and survival of 71% after the treatment and 65% at puberty (Balasubramani 2006). Kavumpurath and Pandian (1994) also reported 36 % mortality of the fighter fish, which were administered 8 µg NE•g^{-1} diet for 40 days. Clearly, the fighter fish is more sensitive to NE than the zebrafish. Hence the study warrants that the response of the zebrafish as a model fish to understand the effects of a steroid (Orn et al. 2000; Peterson et al. 2001; Hill et al. 2005) may not *in toto* be applicable to fishes like the fighter fish.

At a concentration of 1,000 µg•l^{-1}, the NE ensured normal growth in the males only. In all the other tested concentrations, it suppressed the growth, especially among females. At the same concentration of 1,000 µg•l^{-1}, the NE was shown to be a growth suppressant in the *B. splendens* (Balasubramani 2006); its suppressing effects were more pronounced among the female fighter fish. Unfortunately, Varadaraj (1990), who showed that the oral administration of 3µg NE•g^{-1} diet led to the production of 100% masculinized tilapia *O. mossambicus* fry, which weighed 40% more than the control, has not made a life long study. But it is now known that as the age of the treated fish advances, the accelerated growth is normalized and even reduced (Pandian and Sheela 1995). For instance, George and Pandian (1998) estimated the growth of *Poecilia sphenops*, treated with different doses of MT at the age of 3, 6, 9, 12, 15 and 18 months; relative growth was enhanced in the 3-month old treated individuals with increased steroid dose up to pre-optimal level for sex reversal, beyond which the increase in relative growth began to diminish; notably growth was consistently suppressed in the 18-month old individuals, irrespective of treatment intensity.

To the NE treatment, reproductive activity of the male fighter fish is also more sensitive than the zebrafish; for instance, the treatment postponed the age of maturity of the fighter fish from the 110^{th} dph to 168^{th} dph, against only one day in the zebrafish; the fighter fish also extended the inter-milting period from 5 to 28 days, while the zebrafish did it from 5 to 6 days only. Likewise the hatchability of the eggs fertilized by the treated fighter fish was reduced from 91 to 68%, while that of the zebrafish was from 90 to 71% (Balasubramani and Pandian 2008b).

Conversely, the reproductive performance of the treated but persisting female zebrafish and fighter fish suffered almost equally; for instance, they postponed the age of puberty by 43-50 days, and extended the inter-spawning period by 22-24 days. Likewise they also suffered reductions of 15-17% cumulative fecundity and 10-12% progeny production. Incidentally, Nash et al. (2004), who also subjected the zebrafish to life long exposure to 5 ng ethynyl estradiol (EE_2)•l^{-1} reported that the females produced eggs, but none were viable until 14 hrs after fertilization. Other previous workers like Ensenbach and Nagel (1997), Diekman et al. (2004) and Balasubramani and Pandian (2008a), who have made life long study on the effects of pesticides, which mimic estrogen, predicted the imminent extinction of the zebrafish.

The earliest cytogenetic studies of Rishi et al. (1977) and Sharma et al. (1998) indicated that in zebrafish, the female is heterogametic. Traut and Winking (2001) on the other hand failed to identify the sex chromosomes with C-banding, chromomycin A3 or replication banding; in pachytene and diakinesis, none of the 25 bivalents displayed signs of morphological or molecular sex chromosome differentiation (see also Wallace and Wallace, 2003). However, homogamety or heterogamety in many fishes was recognized from genetic studies by progeny testing (Pandian and Koteeswaran 1998). The reported values of the sex ratio of the zebrafish indicates the preponderance of males (58♂ : 42 ♀; 60 ♂ : 40 ♀, Fenske et al. 1999; 68 ♂: 32 ♀, Orn et al. 2000; 56 ♂ : 44 ♀, Vaughan et al. 2001; 69 ♂ : 31 ♀, Brion et al. 2004). Apparently, the sex ratio of the zebrafish is biased towards male. Streisinger et al. (1981) also reported a high frequency of males in clones derived from homozygous female zebrafish. Kavumpurath and Pandian (1990) recorded that all of the triploid zebrafish were males. Clearly, the genetic

mechanism of sex determination of the zebrafish is yet to be known. Hence, for the present it is chosen to designate the phenotypic female and male as the presumed 'homogametic' (X^1X^2) and presumed 'heterogametic' (X^1Y^2), respectively.

Treatments at doses higher than 800 $\mu g \bullet l^{-1}$, the NE generated and ensured the survival of 'homogametic' male zebrafish. Kirankumar and Pandian (2002) likewise recorded that the fighter fish treated at an optimal dose of MT generated and ensured the maximum survival of 'homogametic' males. However, this publication is the first to show that the 'homogametic' males (X^1X^2) could not induce the females to spawn as many eggs as that of 'heterogametic' males (X^1Y^2).

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