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Determination of Age of *Oreochromis niloticus* Fry After Hatching for Efficiency of 17-α Methyltestosterone on Sex Reversal

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Abstract: ***The aim of this research was to determine age of Oreochromis niloticus fry after hatching for efficiency of 17-α methyltestosterone on sex reversal. The study was carried out in Zoology Laboratory, University of Eldoret. Fish were seined from University of Eldoret fish farm and those brooding eggs and fry were selected. Fry were fed using the feed containing 17 α methyltestosterone for varying periods of days. The period of exposure to hormone feed were distributed into 21 days, 30 days, 42 days and 60 days. And at the end of each period, the fry were then sampled in three replicates of ten fry for dissection and microscopic examination. The data was statistically analyzed using Two-way Analysis of Variance and correlations, Statistical Programme for Social Scientists and Excel Statistical package. In all the treatments, no pure females were observed but intersex of varying number of ova was observed. The yolk sac fry from the incubator had 100% conversion efficiency into males both at 21 and 60 days of exposure to hormone, while other groups did not attain 100% conversion into males.***

Keywords: Oreochromis niloticus,fry,hatching ,17-α methyltestosterone, sex reversal

1. Introduction

The culture of *O. niloticus* can be traced to ancient Egyptian which dates back to over 4000 years (7, 11). Evidence is demonstrated by pictures showing ornamental fish in ponds. There is significant worldwide distribution of tilapias. *Oreochromis mossambicus*, distribution occurred during the 1940s and 1950s, while that of the more desirable *O. niloticus* occurred during the 1960s up to the 1980s (7, 14). *Oreochromis niloticus* from Japan was introduced to Thailand in 1965, and from Thailand they were sent to the Philippines. *Oreochromis niloticus* from Cote d'Ivoire were introduced to Brazil in 1971, and from Brazil they were sent to the United States in 1974. In 1978, *Oreochromis niloticus* was introduced to China, which leads the world in tilapia production and consistently produced more than half of the global production in every year from 1992 to present (7). The uncontrolled breeding of tilapia in ponds, which led to excessive recruitment, stunting and a low percentage of marketable-sized fish, dampened the initial enthusiasm for tilapia as a food fish. The development of hormonal sex-reversal techniques in the 1970s represented a major breakthrough that allowed male mono-sex populations to be raised to uniform, marketable sizes (3). In addition, research on nutrition and culture systems, along with market development and processing advances, led to rapid expansion of the industry since the mid 1980s. Several species of tilapia are cultured commercially, but *O. niloticus* is the predominant cultured species worldwide (7, 4).

The breeding process starts when the male establishes a territory, digs a craterlike spawning nest and guards his territory. The ripe female spawns in the nest, and immediately after fertilization by the male, collects the eggs into her mouth and moves to quiet and safe places for mouth brooding. The female incubates the eggs in her mouth and broods the fry after hatching until the yolk sac is absorbed. Incubating and brooding is accomplished in 1 to 2 weeks, depending on temperature (7, 10). After fry are released, they may swim back into her mouth if danger threatens. Being a maternal mouth brooder, the number of eggs per spawn is small in comparison with most other pond fishes. Egg number is proportional to the body weight of the female (14). A 100 g female may produce about 100 eggs per spawn, while a female weighing 600-1 000 g can produce 1 000 to 1 500 eggs (19). The male remains in his territory, guarding the nest, and is able to fertilize eggs from a succession of females. If there is no cold period, during which spawning is suppressed, the female may spawn after every 2 to 3 weeks. While the female is brooding, feeding ceases. Nile tilapia can live longer than 10 years and reach a weight exceeding 5 kg (7, 18). The aim of this research was to determine age of *Oreochromis niloticus* fry after hatching for efficiency of 17-α methyltestosterone on sex reversal.

2. Techniques for Mono-Sex Production

Techniques to produce all-male fingerlings are now an established part of tilapia culture and the use of mixed-sex groups has almost entirely been abandoned (19). Mixed-sex culture was tried in many tropical countries, but even good quality stock that matured late in the wild started reproducing at an early age in aquaculture ponds, and commercial culture failed time and again. Various methods of sex control including, hand-sexing, hybridization and genetic manipulations sex reversal by steroids like androgenic hormones (20).

3. Hybridization

Initial attempts used closely related species such as *Oreochromis hornorum*, *O. niloticus*, *O. aureus* and *O. mossambicus* crosses. Some of these hybrids produced nearly 98% male fish, but hatchery production of large

numbers of fry was inconsistent and unreliable (18). Many *Oreochromis* species utilized in aquaculture were extensively introduced outside their native range in Africa. Given their recent evolutionary radiation, these species hybridize easily, posing a threat to the integrity of local adaptation, (17).

Interspecific hybrid fish have been produced for aquaculture and stocking programmes to increase growth rate, transfer desirable traits between species, combine desirable traits of two species into a single group of fishes, reduce unwanted reproduction through production of sterile fish or mono-sex offspring, take advantage of sexual dimorphism, increase harvest ability, increase environmental tolerances, and to increase overall hardiness in culture conditions (28).

4. Sex Determination

Among mammals sex is usually defined by the presence or absence of the sex specific chromosome Y. In many, but not all, fish species there is also a chromosomal background to sex determination. Several fishes, including most salmonids, have heterogametic males and homogametic females, similar to the mammalian XY/XX-system (32; 33; 23). Other species, such as *Poecilia*, have homogametic males and heterogametic females (ZZ/ZW), which also is the case for birds (36). Some species of the Poecilid platyfish *Xiphophorus*, utilize a system with three sex chromosomes (11). In yet other species sex determination is influenced by environmental factors such as the temperature surrounding the developing embryo (5). Hermaphroditism is also a common feature of several fish species. Several studies have shown that species with genetic sex determination can be directed to produce genetically sex reversed offspring. This is accomplished either by treating the fish with hormones, which can induce sex reversal in synchronous hermaphroditic fish (30) and masculinization/ feminization in gonochoristic species, or by incubating embryos in certain temperatures or pH (3). The proportion of males usually increases with temperature whereas lower temperatures favour females. In the case of pH, species differences have been observed. There are few studies of sex determination in fish and the genetic mechanisms behind sex determination in fish remain largely unknown.

Cichlid fishes of the East African Great Lakes Malawi (LM), Victoria (LV), and Tanganyika (LT) are a prime model system in evolutionary biology and provide an exceptional opportunity to study organismal diversification (13). As for the majority of fish species, the triggers of sex determination in cichlids are largely unknown. Yet, it becomes clear that also in this group various mechanisms exist, including genetic systems and environmental triggers such as water pH and temperature (4). The known genetic factors in cichlids include, for example, sex determination via B-chromosomes (37) and male and female heterogametic sex chromosome systems, with the possibility of both systems co-existing within a single species (27). The best-studied cichlid species with respect to sexual development is the Nile tilapia (*Oreochromis niloticus*), a member of a more basal lineage, widely distributed in rivers and lakes of Africa. The Nile tilapia has an XX-XY sex-determining system, which can substantially be influenced by temperature (4). For this species, expression profiles of key genes of sexual development are available (10) which is not the case for other cichlid species such as the radiating lineages in East Africa.

Fishes have the most plastic system of germ and somatic cells in comparison with other animals. For them, the plasticity is maintained throughout the life cycle. It describes the impact on the process of such factors as temperature, pH, and population density. Temperature sex determination (TSD) at fish is less common than previously thought. The effect of estrogen acting through estrogen receptor (ER) directly or indirectly regulates P450arom and Anti-M¨ullerian hormone (14).

The brain-pituitary-gonad (BPG) axis is the key regulator of sexual maturation. Neuron stimulation of the brain leads to stimulation of the pituitary through gonadotropin releasing hormone (GnRH) which releases relevant hormones, such as follicle stimulating hormone (FSH) and luteinising hormone (LH) into the blood plasma for transport to the effector tissue. In the testes LH induces the production of testosterone which then affects various aspects of male physiology, secondary sexual characteristics and behavior (9).

5. 17α-Methyltestosterone (MT) Mode of Action and its Advantage on Sex Reversal

The synthetic steroid 17α- methyltestosterone is a male specific hormone commonly used to induce sex reversal in teleost fish. 17α-Methyltestosterone (MT) is a synthetically produced anabolic and androgenic steroid hormone; i.e. it promotes both muscle growth and the development of male sexual characters (1).

Androgens are commonly applied for hormonal stimulation of growth or sex reversal in fish (6). Among the androgens, MT is most commonly used. It is easily absorbed, does not accumulate in fish body and is readily excreted (29).

Sex reversed tilapia showed a better growth rates than normal because administration of androgen have both an androgenic and anabolic effect. There are several studies comparing the growth of sex reversed, near all male populations, to that of a mixed sex population after hormone treatment showed the improved growth of sex reversed fish than non-treated because the presence of females reduces the growth rate due to their slower growth rate or reproduction (16). Commercial tilapia production generally requires the use of male monosex populations. Male tilapia grows approximately twice as fast as females (7).

Oral Hormonal Administration

Oral administration procedure of sex reversal involves feeding fish with hormone treated diet. Fish exposure to androgens usually occurs through dietary treatment, and the most commonly used androgen is 17 α – methyltestosterone (MT). Feed preparation has been described by Popma and Green (25; 19). Feeds that contain 25 to 45% protein are recommended, although a lower level of 20% protein feed has been used successfully (27). Androgen-treated feed is

prepared by mixing androgen that has been dissolved in solvent, usually 80 to 95% ethanol with fine grounded feed or the hormone treated feed may be prepared by spraying a solution of androgen in 95% ethanol onto the feed. Mixing the components of feed and hormone minimizes atomization of androgen solution, which reduces the risk of contamination of workers. However, the spray method is advantageous in that the volume of solvent can be greatly reduced. In both cases, the alcohol is allowed to evaporate and the dried feed stored in a cool, well-ventilated area or refrigerated until used; good air circulation around the complete feed container helps maintain feed quality during storage. Because MT is photosensitive, pure MT should be protected from sunlight, and treated feed should not be dried or stored exposed to direct sunlight. Feed that was prepared with MT and stored exposed to light was found to be ineffective in sex inversion of *O. mossambicus* (35).

The age of *O. niloticus* fry is an important factor that influences the efficiency of sex reversal efficiency and should be considered. Treatment duration of 3 to 4 weeks consistently produced male tilapia populations comprising ≥95% males, while periods that exceed 4 weeks did not further improve the efficacy of the treatment (31; 22; 17). However, at lower water temperatures of about 20±2^{0}C, increasing the duration of treatment from 20 to 40 days increased the efficiency from 69 to 95% males (15). Treatment with higher doses of MT for 19 or 28 days did not result in successful sex reversal (18; 21). Ridha Lone (27) reported no significant differences in tilapia fry survival, and final weights after a 38 day trial where fry were fed androgen-free diet containing 30, 50, and 70 mg MT/kg. From the ongoing it is clear that 100% all male population has not been attained. For successful culture of tilapia, there must not be females in the population because presence of a few female would negate the gains achieved in sex reversal. The current study was aimed at determining the age and duration of hormone exposure which would lead to 100% conversion efficiency of 17-α methyltestosterone on sex reversal of Nile tilapia**.**

6. Materials And Methods

6.1 Study Area

The study was carried out in Zoology Laboratory, University of Eldoret. The University is located at Latitude of 00^0 and Longitude 035^0, and 2154m above mean sea Level.

6.2 Sources of Experimental Fry

Fish were seined from University of Eldoret fish farm and those brooding eggs and fry were selected. The fry and eggs were transported to the laboratory for further processing. The fry were placed in the aquarium while the eggs were incubated in incubation jars until hatching.

6.3 Hormone Preparation

The hormone feed was prepared by weighing the desired quantity of hormone dissolving it in 95% ethanol and spraying it on the feed. Methyltestosterone was incorporated at 40mg per kilogram of feed. The feed was then dried under shade and stored in a well ventilated container in a cool place.

6.4 Feeding

Fry were fed using the feed containing 17 α-methyltestosterone for varying periods of days. The period of exposure to hormone feed were distributed into 21 days, 30 days, 42 days and 60 days. And at the end of each period, the fry were then sampled in three replicates of ten fry for dissection and microscopic examination.

6.5 Microscopic Examination

Sex of fry was determined through microscopy using compound microscope. Identification was aided by staining the gonads using aceto-carmine. A readymade solution of aceto-carmine was purchased and used to stain the gonads in the staining jar for six hours. The stained gonads were mounted on new microscopic slides and covered with a cover slip. The microscopic slides and cover slips containing stained gonads were then observed under compound microscope using oil immersion at 100x objective power, and microscopic slide photos were taken using digital camera mounted on a microscope.

6.6 Statistical Analysis

The data was statistically analyzed using Two-way Analysis of Variance and correlations, Statistical Programme for Social Scientists (version 11.5) and Excel Statistical package.

7. Results

Microscopic Observation for Sex Determination of *O. Niloticus*

The results of microscopic observations are shown in Table 1. For all the treatment no pure females were observed but intersex of varying number of ova was observed. The number of ova in the intersex fry ranged from a minimum of one to a maximum of eight. After 21 days of hormone exposure of fry from brooding mothers which had absorbed the yolk sac were 50% converted into males and when the period was increased to 42 days, the conversion efficiency into males increased to 80%. Fry from the mouth of brooding mothers which had yolk sac and exposed to hormone for 21 days resulted into 60% conversion efficiency. When the period for same fry was extended for 30 days, the conversion efficiency into males increased to 70%. The yolk sac fry from the incubator had 100% conversion efficiency into males both at 21 and 60 days of exposure to hormone.

Table 1: Microscopic observation for sex determination of *Oreochromis niloticus*

Category (Age)	Hormone period in days	No. of Males	No. of Females	No. of Intersex	Total
Fry from mouth having no yolk sac	42	9	0	1	**10**
		10	0	0	**10**
		9	0	1	**10**
		Total			**30**
	21	7	0	3	**10**
		9	0	1	**10**
		9	0	1	**10**
		Total			**30**
Fry from the mouth with yolk sac	21	9	0	1	**10**
		8	0	2	**10**
		9	0	1	**10**
		Total			**30**
	30	8	0	2	**10**
		9	0	1	**10**
		10	0	0	**10**
		Total			**30**
Yolk sac fry hatched in incubators	60	10	0	0	**10**
		10	0	0	**10**
		10	0	0	**10**
		Total			**30**
	21	10	0	0	**10**
		10	0	0	**10**
		10	0	0	**10**
		Total			**30**

The results from Pearson correlation are shown in Table 2. According to Pearson Correlations, category and sex of fish was significant at (P<0.05), as showed in table 2. Hormone period and category (age) was highly significant at (P<0.05). Pearson correlation showed that there was no significance between hormone period and sex of fish. Category was significantly correlated to period of exposure. The interaction between the category of fry and period of exposure was significant (P<0.05). Category was also significantly correlated to sex reversal inversion. The period of exposure to hormone was not significantly correlated to sex inversion.

Table 2: Pearson Correlations of sex of fish, category and period of exposure to hormone

		Category	Period of exposure to hormone	sex of fish	Temperature
Category	Pearson Correlation	1	-.242(**)	.174(*)	-.008
	P-value	.	.001	.019	.912
	N	180	180	180	180
Period of exposure to hormone	Pearson Correlation	-.242(**)	1	-.132	-.009
	P-value	.001	.	.077	.904
	N	180	180	180	180
sex of fish	Pearson Correlation	.174(*)	-.132	1	.014
	P-value	.019	.077	.	.852
	N	180	180	180	180
Temperature	Pearson Correlation	-.008	-.009	.014	1
	P-value	.912	.904	.852	.
	N	180	180	180	180

** Correlation is significant at the 0.01 level.* Correlation is significant at the 0.05 level.

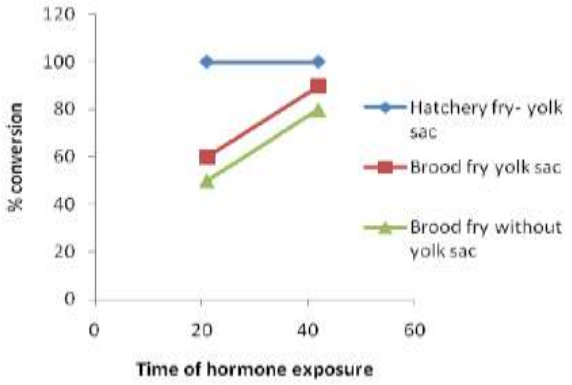

Figure 1: Interaction between fry category and hormone exposure

8. Discussion

In the present study, an inversion rate of 100% was observed in fry that were hatched from eggs in the laboratory. Both yolk-sac-fry and those lacking the yolk from the mouths of brooding mothers did not achieve 100% sex inversion. Further, no pure females were observed during microscopic examination and all departures from pure males were inter-sex individuals. This observation suggests that all fry had access to the hormone treated feed in one way or the other. Others authors found that producing a monosex population of *O. niloticus* for aquaculture is high priority since males have a higher growth rate as compared to females and their observations revealed male percentage above 90% but less than 100% Utete and Victor (34). The results as indicated by others here meant that the methods used were crude and need refinement. This may lead to lack of pure female. Most experimental evidence only partially supports a monofactorial model for sex determination in tilapia; autosomal and environmental factors also are thought to influence sex determination (8).

The present study revealed that after 21 and 42 days of hormone exposure of *O. niloticus* fry from brooding mothers without the yolk sac produced both males and intersex. *O. niloticus* fry from the mouth of brooding mothers which had yolk sac and exposed to hormone for the same period resulted into more males and less intersex. However, the extension of time exposure to the hormone to 60 days slightly reduced the number of intersex fry. It is likely that from this result, the fry with yolk sac performed better than those without. This is because the fry without yolk sac were higher age as compared to fry with yolk sac. It is well established in the literature that fry of lower age respond better than an advanced aged fry (24). It is more likely that the hormone was absorbed by yolk which was intern a simulated by the fry since the sole nutrition of the fry dependent on the yolk. This result agrees with (2) on their results which showed that yolk sac fry exposed for a longer period to hormone feed led to higher rate of male conversion with very few intersex observed.

The result of the present study indicated that the yolk sac fry from the incubator had 100% conversion efficiency into males both at 21, 42 and 60 days of exposure to hormone. In this set up, eggs were incubated at a higher temperature which decreased embryonic development time and the same time, the swim-up fry had the first encounter to only hormonal feed. Given that sex determination conversion efficiency is dependent on age of fry; such fry are relatively younger and are more likely to be transformed with higher conversion efficiency into males. Gonadal tissue differentiation is presumed to occur between 8 to 25 days post-hatch, which is influenced by environmental conditions (8). In the present study, fry were exposed to hormone one day post-hatch. It is likely that masculinization hormonal stated its influence before the on-set of gonadal differentiation thus culminating into 100% conversion efficiency. This result did not conform to any result of other authors (22). The reason for the non conformity was because the current experiment was carried out within a confined aquaria environment which exposed all fry to hormone feed.

9. Conclusion

The present study demonstrated that it is possible to achieve 100% all male mono-sex population. However to achieve this result fry must not be beyond the lower limit of 8 days post-hatch before exposure to hormone. For lower age fry conversion was independent of duration of exposure whereas for fry beyond 8 days post-hatch, the rate of male conversion was positively related to duration of exposure. Incubator hatched fry gave 100% male conversion rate whereas those snatched from the mouth did not attain 100% conversion efficiency at the duration of the experimental examination. Fry which had yolk sac from mouth brooding mothers and those hatched from eggs in the laboratory differed in their rates of conversions despite both having yolk sac they yielded different results in terms of male conversion efficiency. It was found that temperature was a key factor for faster egg and fry development.

10. Recommendations

The age of fry is highly significant for efficiency of 17 α-methyltestosterone on sex reversal and a target below 8 days post-hatch could be taken into account. Sex reversal should start with fry of below 8 days old after hatching and duration of exposure for twenty one (21) days would be sufficient.

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