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Determination Of The Most Appropriate Day To Introduce 17α-Methyl Testosterone Feed To Swim-Up Fry For Effective Sex Reversal In Nile Tilapia.

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Abstract: *Mono-sex culture of male Nile tilapia is preferred because of their faster growth than females. This study was conducted to investigate fish growth performance and sex reversal efficiencies by oral MT administration. The Feeding on the hormone-treated diets was initiated at 2, 6 and 10 days after the yolk-sac disappearance. Three control treatments were set-up for each treatment where fry were not exposed to MT. Water was replaced after every two days. Fry were fed 5% body weight three times daily at 10 am, 12 noon and 4pm. At the end of each experiment all fingerlings were dissected for gonadal sex determination using the aceto-carmine squash method. All the treatments containing MT produced significantly ($p<0.05$) higher proportion of males compared to the control treatment. The mean proportions of males from 2 and10 day-post hatch treatments were similar but differed significantly ($p<0.05$) from the day 6 post-hatch treatment. The highest percentage of 92.2 males was obtained from day 6 while the lowest 77.7% was obtained from day 10. The highest growth rate ($0.21\pm0.08\ day^{-1}$) was observed in day 6 and the lowest ($0.13\pm0.02day^{-1}$) in the control treatment. In conclusion, MT influenced the sexual differentiation of in O. niloticus in favour of males. Day six post-hatch was the most effective timing in terms of male conversion rate. Oral feeding of the hormone treated diets enhanced post-fry growth. The day 6 post-hatch is highly recommended for the effective sex reversal of Nile tilapia.*

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I. Introduction

Sex differentiation in tilapia is a labile process that allows sex inversion in several species. The inherent capacity of the germ cells to differentiate into oocytes or spermatocytes constitutes a key factor allowing for functional sex inversion. The *Oreochromis niloticus* (Linnaeus, 1758) is one of the most important species in tropical and sub-tropical aquaculture (FAO, 2007). The fish provides one of the major sources of animal protein and income in most parts of the world (Sosa *et al*., 2005). It is currently ranked second only to carps in global aquaculture production and is likely to take over from carps in the 21st century (Ridha, 2006). Tilapia grows and reproduces in a wide range of environmental conditions and is able to tolerate stress induced by handling (Tsadik and Bart, 2007). The success of the culture techniques applied in tilapia farming depends on several factors and determination of suitable culture technique can be quite complex (Graaf *et al.,* 2005). Various traditional and non-traditional tilapia farming methods have been adapted in different countries in accordance with the socioeconomic and ecological conditions of a particular place (Lèveque, 2002). However, the efficient production of tilapia in a wide range of environment conditions has been countered by some undesirable consequences. The common problems in tilapia culture are the reduction of growth rates at the onset of sexual maturity and precocious reproduction, leading to undesirable sizes of small fish production (Lèveque, 2002). Another problem is that females, which constitute one half of the culture population, grow much slower than males. Sex-specific differences in growth are significant in *O. niloticus* where males grow significantly faster, larger and more uniform in size than females (Bwanika *et al.*, 2007). There are a number of ways to control reproduction in populations of tilapia. One of these strategies is the culture of all-male tilapia population (Phelps and Popma, 2000). The desirability of mono-sex male populations of tilapia is well established for increased production potential and low management requirements (Pillay, 1993; Beardmore *et al.,* 2001; El-Sayed, 2006). The development of strategies for generation of mono-sex populations remains a critical objective for the tilapia culture industry. *Oreochromis niloticus* has an XX (female) and /XY (male) chromosome sex determination system (Baroiller *et al.,* 2009), but the process of sex differentiation being labile renders a possibility for sex reversal in the species (Devlin and Nagahama, 2002). Several techniques have been adopted for production of mono-sex (all-male) populations of tilapia (Phelps and Popma, 2000), and hormonal sex reversal of tilapia has been an active area of research for the past three decades (Pandian and Varadaraj, 1988; Gale *et al.,* 1999; Carrasco *et al.,* 1999*;* Afonso *et al*., 2001). Oral administration of exogenous sex hormones

before the differentiation of primal gonadal cells can cause reversal of phenotypic sex (Smith and Phelps, 2001; Bhandari *et al*., 2006). The importance of mono-sex tilapias has been established in many commercial contexts., but the specific day to start feeding the fry on MT-feed is not well established for effective sex reversal. Moreover Strussman and Nakamura (2003), pointed out that the mechanism of action of exogenous steroids during sex differentiation is not sufficiently clear. An earlier study by Chakraborty *et al*, (2007) showed that treating 3 days old fry with a synthetic male hormone 17α- methyl testosterone (17α-MT) at a treatment regime of 10 mg/kg food for 30 days produced almost 100% all-male mono-sex tilapia population. However, among the doses, timing and different applications modes have always given inconsistent results with similar and /or different tilapia species (Smith *et al*., 2001; Bhandari *et al*.,2006). Therefore a study to investigate the same is relevant.

II. Materials And Methods

Area of Study

This study was set and carried out at the zoology laboratory-I, for aquaculture research, University of Eldoret, Uashin Gishu County, Kenya. Uasin Gishu county lies between longitudes of 34˚ 50 east and 35˚ 37 west and latitude 0˚ 03 south and 0˚ 55 north (UGIDP,2013). The county shares common borders with Trans Nzoia county to the north, Elgeyo Marakwet county to the east, Baringo county to the south east, Kericho to the south, Nandi County to the south west and Kakamega County to the north west. It covers a total area of 3345 square kilometers(UGIDP,2013) The County enjoys two rainy seasons with an annual rainfall ranging between 900 to 1200 mm. Sited on a plateau, the county has a cool and temperate climate, with annual temperatures ranging between 8.4°C and 27°C. The wettest season in Uasin Gishu County is experienced between the months of April and May while the driest season comes between January and February. It began on January and terminated on May, 2014.

Table 3.1c: The oral feeding on 6mgkg^{-1} MT treated diet to post-hatch (swim-up) fish

Treatment Tank	Dose MT(mgkg^{-1})	Feed onset day
O	0	0
J	6	2
K	6	6
L	6	10

Plate 3.1: The arrangement of the aquaria tanks used in culture of fish during culturing period source:Obonyo(2014)

3.3 Egg collection

Nile tilapia (*Oreochromis niloticus)*, eggs used in the present experiment were purchased and collected from fish farm, Department of Fisheries and Aquatic Sciences University of Eldoret in the month of January, 2014. About 15 adult Nile tilapia (*O. niloticus*) were harvested daily using seining fish-net from ponds whose water had been changed a week earlier (Plate3.2**)**. All females of the harvested fish were checked in the mouth during each egg collection. Once eggs were noted and obtained, it was recorded as the first day of mouth-brooding. Eyed-eggs were collected from the mouth of the brooding female tilapia late morning (10.00am-12.00am). Eggs were pooled and stocked in 10L capacity plastic containers at an average stocking density of 3000 eggs per container and were transferred to the laboratory. Females carrying eggs in the buccal cavity could be identified by an unusual unique persistent shut of the mouth even when removed out of water.

Plate3.2: Seining method of fishing as used during fish harvesting for the collection of eggs
Source:Obonyo(2014)

3.4 Incubation

The fertilized eggs were rinsed with clean water, counted and then kept in an indoor laboratory condition in 10 liters transparent buckets with water for four hours to acclimatize before transferring them to the artificial incubators (round bottomed flask 1000 Milliliter each) (Plate3.3). They were then sorted and distributed into the incubator at a density of 300 eggs /flask. The flasks were then submerged in a warm water bath, maintained at a temperature of 30^{O}C by a thermostat heater, which ensured that water in these flasks were raised and maintained at 29 ± 0.5^{O}C, with a normal photoperiod of 12 hours light and 12 hours darkness. All eggs were incubated in clean freshwater provided with sufficient and constant aeration to control the ammonia concentration levels and facilitate continuous movement of eggs in the water column.

Eggs were inspected daily and unfertilized or dead eggs (which changed the color from brown to white) were removed as their presence may cause contagious infection and spoil of other eggs thus loss of the whole spawn. Incubation continued until hatching after about 2-4 days (Arriesgado *et al*., 2011). After 2 days of incubation the fertilized eggs hatched (Mateen, 2007). The procedure was repeated three times until enough hatchlings were collected.

Fry were reared in the incubation system for a period of 4 days till the swim-up stage. Hatching percentages and fry survival was recorded for all the batches. Finally, 120 yolk-sac fry per spawn were randomly sampled for distribution in the aquaria tanks of 40.5L each and dimensions of length (45cm) by breadth (30cm) by height (30cm) where they were reared for the next four months.

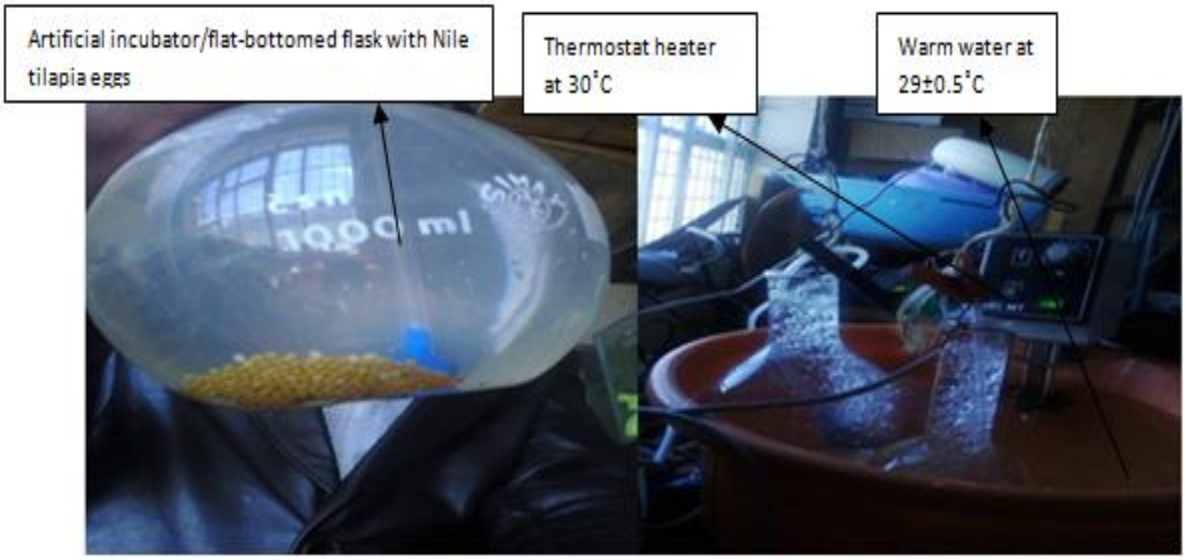


Plate 3.3: The artificial incubator and incubation set up
Source: Obonyo (2014)

3.5 Preparation of 17αMethyl-testosterone solution

A stock solution was made by dissolving 6mgL^{-1} in 10% ethanol as a carrier liquid since 17α-Methyl testosterone is sparingly soluble in water. The 17α-Methyl testosterone is a white synthetic androgen which is in a powder state. 6mg of the powder was added into 10ml ethanol. The solution was shaken repeatedly to ensure complete dissolution of hormone hence the homogeneity of the solution. The 6mgL^{-1} hormonal concentration (HC) was prepared from stock solution. During the treatment, the solution was topped up with distilled water to 1000ml. Finally the hormone solution was safely kept in the laboratory at room temperature (22±2˚C) till use in either immersion or mixed with feed.

3.6 Immersion of egg and yolk-fry in 17α-MT solution

The immersion technique was carried out in two developmental stages of fish at egg and yolk-sac fry. Immersion was carried out using plastic buckets of 2L each, labeled 2 hours, 4 hours and 6 hours. The containers were suspended in aquaria tanks measuring (45cm × 30cm × 30cm). Aerators were provided in each container to facilitate the continuous supply of oxygen to the egg or yolk-sac fry in the water, and to allow movement of the eggs or yolk-sac fry in the water column to prevent the prolonged contact among themselves and with the surface of the incubator which could otherwise lead to their death and rot.

3.*6.1 The egg immersion technique*

Fertilized eggs were examined, sorted and immersed in the 6mgL^{-1} solution at different immersion time of 2 hours, 4 hours and 6 hours according to Pifferer & Donaldson (1993). Control group was only immersed in 1000ml of 10% ethanol which did not contain MT hormone (Celik *et al*.,2011). The hormone bathed eggs were then removed from the solutions, rinsed and set in the incubators for hatching.

3.6.2 The yolk-fry immersion technique

Three-day old post-hatch (dph) yolk-sac fry were collected from the non-hormone treated batch of eggs were immersed in 6mgL^{-1} 17α-Methyl testosterone hormone at different immersion time (IT) of 2 hours, 4 hours and 6 hours (Pifferer and Donaldson., 1993). After immersion, treated yolk-sac fry were rinsed with clean tap water and transferred into glass-aquarium tanks of 40.5L each. The room temperatures were regulated by room heater to about 28±2^{O}C for optimum growth in each tank where they were placed and reared for the entire research period.

3.6.3 The oral feed technique

In the oral feed experiment the post-hatch fry from none-hormone treated batch of eggs were randomly distributed into aquaria tanks of 40.5L, at a stocking density of 60 fry per treatment tank (Phelps and Popma, 2000). Immediately the yolk-sac disappeared, it was recorded as the first day of yolk-sac disappearance. Oral feed treatments were varied in terms of days to start feeding the fry on hormone-treated diet. Therefore the feeding was initiated 2 days, 6 days and 10 days after the day yolk-sac first disappeared. Control treatment was fed on normal diet right from the first day yolk-sac disappeared to the end of research period. All the aquaria

tanks were provided with aerators in order to saturate water with oxygen. Each treatment group was replicated three times with a control each. The average weight of fry at the beginning was (0.013±0.007g/fry).

3.7: Feed preparation

The diet samples (fresh water shrimp, cotton seed and wheat bran) were ground into fine-powder. Fish-feed in this experiment is formulated by taking accurate measure of the powdered samples by use of a Citizen scale CY204, Poland, precission:0.0000 g in the proportion of 0.64g wheat bran, 0.24 cotton seed and 0.12 shrimp to make a Kilogram of the feed. This was sprayed by ethanol solution without the MT hormone and labeled normal diet.

The hormone treated diet was prepared as described by Killian and Kobler. (1991). First a kilogram of normal diet was prepared. Hormone-feed preparation was done with dosage of $6mg^{-}kg^{-1}$ as the standard dosage protocol in oral feeding (Okoko.,1996). 17α-MT hormone-treated diet was prepared through ethanol evaporation method (Piferrer *et al*, 1993). The $6mgL^{-1}$ 17α-MT solution was sprayed into the diet and mixed properly after which, the feed was left to dry at room temperature for 12 hours. It was stocked in plastic storage bags and preserved at room temperature (Celik *et al*., 2011).The feeds were then categorized and labeled as either normal or MT-treated diet. The hormone treated diet was used to feed the fish during the grow-out period for 30 days and then substituted with normal feed for the last 3 months.

3.8 Feeding patterns

The fry mean average weight 0.013±0.007 g; mean length 1.25±0.012cm from all the treatments were fed at a feeding rate of 5% of mean body weight (Celik *et al*., 2011) three times at 10.00am, 12.00 noon and 4.00 pm per day for 6 days a week for four months.

3.9 Culture technique

All the aquarium tanks had equal dimensions of 30cm x45cm x30cm (width x length x height), with a maximum water holding capacity of 40.5 liters (Plate3.1). Each tank was filled with tap water earlier preserved in 210 Liters cylindrical plastic container for two days to dechlorinate the tap water. The aquaria tanks were supplied with compressed air via air-stones from air pumps to ensure constant concentration of dissolved oxygen (DO). The temperatures in the tanks were maintained within a range of $28\pm2^{O}C$ with an aid of a room heater and normal photoperiod of 12 hours light and 12 hours darkness.

Rearing, Water Management, Feeding Protocols and Water change started after the designated immersion periods prior to oral administration of diets. Semi-dynamic method for removal of excreta was used every 2 days by siphoning a portion of water from the aquaria tanks and replacing it with equal volume of water. During the rearing period water quality parameters (Temperature, pH, Dissolved oxygen (DO) and Total dissolved solids) in the glass aquarium tanks were measured weekly up to the end of the experiment using YSI ProCom II, ITEM:605404 L/N:12G100510-U.S.A. The fish were reared under this controlled laboratory conditions for four months until they were large enough to be dissected for sex determination.

3.9.1 Data Management and Analysis

Data on sex ratio, fry growth rate were evaluated. The time of development of the fry from the period of immersion up to oral administration of formulated diet was closely monitored. Growth, survival and health condition of the fish were determined every month throughout the experimental period. Manual sex determination of tilapia was made after four months of culture when sex identification was possible.

Numbers of the fry that survived to the end of the experiment were recorded to assess survival rate. Thirty fry (4 months old fish) out of the total fry survived from each treatment were sampled. The final length and weight of the fish were measured by a Citizen scale CY204, Poland, precission:0.0000 gm. Both values were used in calculation of growth parameters. They were then sacrificed and dissected for gonads. The gonads excised using fine forceps. Collected gonads were mounted on a glass slide and few drops of aceto-carmine stain were added. Gonads were lightly squashed with a cover slip (Guerrero and Shelton, 1974). The gonad mounts were examined under a light microscope using magnifications of 100 to 400x (Guerrero and Shelton, 1974). Based on histological evaluations of the gonads, each fish was classified as male (testes), female (ovaries), or intersex fish (ova-testes). An intersex fish was one in which at least one gonad contained both ovarian and testicular tissues simultaneously.

1.Measurement of growth parameters (a)length(cm) by a ruler and (b) weight(g) by a Citizen scale CY204, Poland, precission:0.0000 g

2.(a)gonadal excision and (b)microscopic examination of the gonadal tissues

Plate 3.*4: Measurement of growth parameters, gonanadal excision and microscopicexamination of gonads*
Source: Obonyo (2014)

Statistical analyses of data were performed using means and standard deviations, with the aid of a simple linear regression and a correlation (T-test) in a Stratigraphic trial version16.1.11(32-bit), Microsoft excel spreadsheet for window 2007 and Minitab statistical software version 14. A one-way Analysis of Variance (ANOVA) was used to compare the hatchability, growth, and male production proportions of *O. niloticus* exposed to MT at different IT. Percentage data (i.e. male proportions) were arc-sine transformed prior to Analysis of Variance and testing for significant differences (Srisakultiew *et al*., 2013). The differences among treatments were considered using confidence intervals from the regression analysis. All inferences were statistically significant when the $p \leq 0.05$. (Kamonrat *et al*.,2013).To determine the growth rate, the length component was obtained from calculation of the length–weight relationship of $w=aLb$, *but linearised into* $logW=loga+blogL$, to obtain the allometric size–length relationship with linearity confirmed by polynomial regression, where; a=intercept of the standard length and weight relationship, b=slope or exponent (ß) of the standard length and weight relationship, w=weight (g) and L=standard length (mm). Slopes (ß) between the treatments was calculated by running the linear regression of each treatment separately and resultant slopes used to calculate the Student's t–test.

III. Results

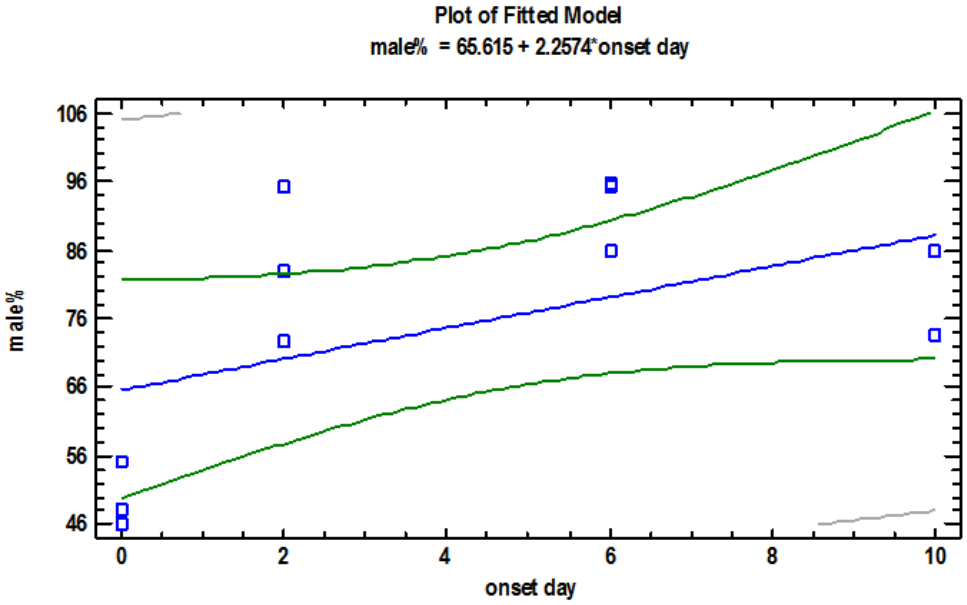
Sex reversal rates varied with the day when the fry were first fed on hormone treated diet (Table 4.6)**.** There was significant difference in percentage of male in *O. niloticus* between the hormone treated diet and the diet without the hormone/control ($p<0.05$). The percentage male increased from day 2 to day 6 after which a declining trend of percentage of male was observed. However, the highest percentage of intersex fish was obtained when the fish were introduced to the hormone-treated diet at day 10. The lowest mean percentage males of 49.6±4.5 was obtained from control experiment while the highest percentage of male of 92.2±3.4 was obtained when the fish were introduced to $6mgkg^{-1}$MT hormone-treated diet on day 6 after the yolk-disappearance. Each hormone treated group gave a mean male/female ratio that deviate significantly from the normal 1:1 ratio obtained from control treatment, with the number of males significantly more than the females. No treatment gave 100% male population of *O. niloticus.*

Table 4.6: Mean percentage proportion of males , females and intersex variation by day of first feeding the fry on the MT-treated diet(95.0% LSD intervals)

Onset day of feeding	N	Male		Female		Intersex		*Significant male productivity
		%	S.E	%	S.E	%	S.E	
0	3	49.6±4.5	4.4	50.3	3.1	0	5.2	a
2	3	83.6±3.5	4.4	5.3	3.1	11.0	5.2	b
6	3	92.2±3.5	4.4	1.6	3.1	6.3	5.2	c
10	3	77.7±3.5	4.4	7.7	3.1	14.1	5.2	b

* Values with same letters are not significantly different (P>0.05)

Linear regression curve (percent male= 65.6 + 2.3*onset day) for the relationship between the sex reversal and interval of feeding onset day (Figure4.4).There was no statistically significant ($p<0.05$) relationship between male% and the day feeding began at 95.0% or higher confidence level (Table4.7).The relationship between sex reversal and day of feeding onset explains only 25.8% of the total variability of the regression model.



IV. Discussion

The treatment days in the present study were within the labile period for sex reversal in *O. niloticus* which ranges from 9-21 days after fertilization of eggs (Baroiller *et al*., 1996). The higher mean male percentages obtained in hormone-treated fish could be attributed to the androgenic effect of MT. These findings were in agreement with the findings of Okoko.,(1996), who obtained 97% males at the dose rates of 60mgkg^{-1} MT which is considered the oral MT dosage in standard protocol (Little *et al*., 1995). An introduction of hormone treated food to a two-day old swim-up fry was too early. At this time, the fish were too young therefore had slower feeding and poor food conversion rate. So little of the MT-treated feed was consumed and assimilated. The highest percentage male at day 6 could be due to the high feeding rates that resulted into high conversion rate (assimilation) of MT-treated food. At this time, the fish were larger enough for faster feeding on MT treated food. At day10, the fry had begun undergoing sex differentiation while some could have completed sexual differentiation. Therefore introduction of MT at this time would only interrupt the sexual differentiation process and forced the sex inversion of the fry (Nakamura and Iwahashi, 1985). Formation of feeding hierarchies (Macintosh *et al.*1985) could also lead to some fish accessing only insufficient MT dose. This explains why the highest intersex fish were observed at day 10. These results supports the alternative hypothesis that sexual differentiation in fish may occur during the early embryonic stages which is supported by findings of (Kwon *et al.*,2001, Tsai *et al.*,2003)

Most intersex fish were obtained from the day 10. The presence of intersex (ova-testes) fish could be attributed to low concentration of MT in the fish that could not occupy all the hormonal binding sites, consequently allowing the estrogen to co-exist with the androgen. Such circumstances led to production of both spermatocytes and oocytes. When the hormone is introduced at some later stage in post-hatch development (by oral feeding) then it is impossible to completely suppress the production of estrogen and therefore both testicular

and oval tissues are formed leading to intersex fish (Nakamura and Iwahashi, 1985). Additionally occurrence of intersex fish could be attributed to late hormonal treatment in which some germ cells had already been committed differentiation towards oogonia. Macintosh *et al.* (1985) reported that occurrence of intersex in fish could be due to the genetic non-responsiveness by some females to steroids. The steroid treatment may induce gonadal cell differentiation directly in the gonad independent of the brain. However if sex inversion was to be controlled by the brain, the entire gonad could be regulated by such a mechanism without missing individual cells (Contreraz Sanchez., 2001). There is possibility that synthetic steroids interact with steroid receptors present in the primordial gonad in order to exert its masculinizing effects (Contreraz Sanchez., 2001). It was also suggested that unavailability of sufficient hormone treated food due to faster growth by some fish to form social hierarchy (Hiot and Phelps, 1993;Pandian and Veradaraj, 1988; Alvendia-Casauay and Carno, 1988). None of the treatments gave 100% inversion to male fish, however if the intersex fish was considered to be males the day 10 gave 100% sex inversion.

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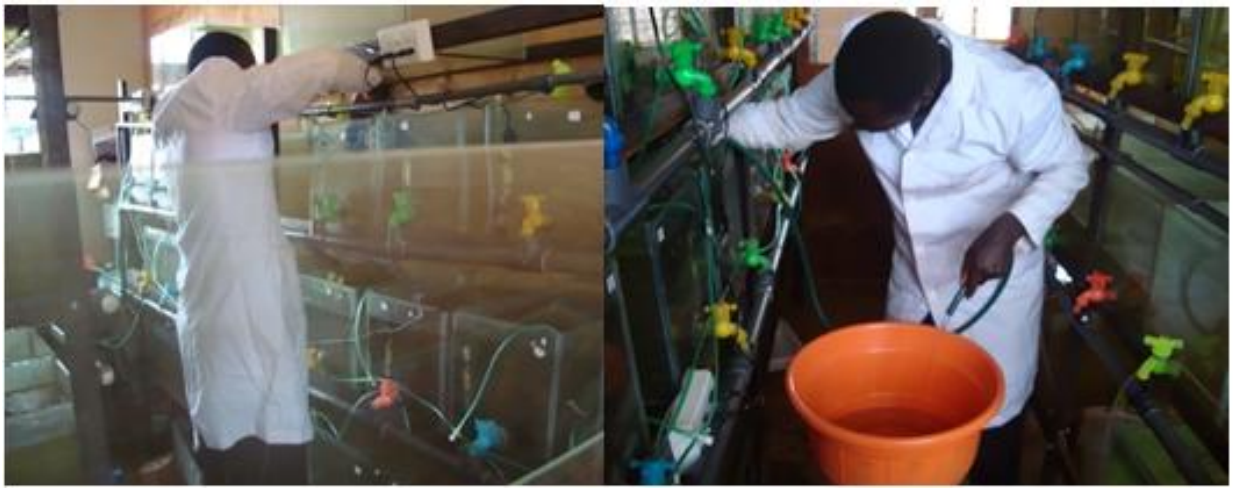
APPENDICES



Appendix i: A photograph showing the siphoning technique of cleaning the aquaria tanlks in the culture system
Source:Obonyo(2014)

Appendix ii: Summery statistics of hatchability of eggs at different IT

immersion time	Count	Average	Standard deviation	Coeff. of variation	Minimum	Maximum	Range
0	3	50.3233	4.52965	9.00109%	45.57	54.59	9.02
2	3	72.8667	4.8758	6.6914%	69.16	78.39	9.23
4	3	89.37	5.40429	6.04709%	83.46	94.06	10.6
6	3	97.57	0.851058	0.872254%	96.83	98.5	1.67
Total	12	77.5325	19.2109	24.7779%	45.57	98.5	52.93

Appendix iii: Water quality parameters variation during the culturing period

Parameter	**Day1**	**Day2**	**Day3**
TEMPERATURE (◦C)	25.9	27.09	27.26
PH	6.88	7.64	7.71
D.O mg/l	5.01	4.33	3.99
SALINITY S‰	0.09	0.11	0.13

Obonyo Nicholus O., "Determination Of The Most Appropriate Day To Introduce 17α-Methyl Testosterone Feed To Swim-Up Fry For Effective Sex Reversal In Nile Tilapia." IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS), vol. 18, no. 10, 2019, pp 01-16.