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Development of All-male Fingerlings by Heat Treatment and the Genetic Mechanism of Heat Induced Sex Determination in Nile Tilapia (*Oreochromis niloticus* L.)

P. O. Angienda, B. O. Aketch and E. N. Waindi

***Abstract*—**Juvenile Nile tilapia subjected to heat treatment at temperatures ranging from 26^0C to 37^0C showed positive correlation (P<0.01) between treatment temperatures and resultant sex ratios, while, survival rate of the fry showed a negative correlation against temperature (P<0.01). The optimal temperature for both sex shift towards males and survival rates was 36±0.5°C, producing male percentage of 86.31 and a fry survival of 65.25. To determine the genetic basis of temperature sex-determination in Nile tilapia, we employed three microsatellite markers (Abur36, Abur100 and UNH846). Abur36 predicted the sex of 95% of the heat induced individuals, suggesting that the locus influence sex ratio and its interaction with temperature result in male biased sex ratio. This locus could turn out to be the major sex determining gene operating in Nile tilapia. These markers could be used in marker-assisted selection to select genotypes that give a higher percentage of males for commercial production.

***Keywords*—**Heat treatment, Microsatellite, Nile tilapia, sex-determination.

I. Introduction

Nile tilapia (*Oreochromis niloticus*) is a popular culture fish due to its economic importance. It has a wide range of distribution in both tropics and temperate regions. It is the most dominant fish among the group of tilapias farmed in sub-Saharan Africa, either for subsistence or export. Cultured *O. niloticus* are easily bred in captivity without complex hatchery equipment or hormonal induction of spawning. They can be bred year round in the tropics. Tilapia farming therefore, if well developed, could provide the needed fish protein as well as monetary gains to the communities in the Lake Victoria region.

The basic problem of aquaculture however, is that a mixed sex, freely breeding *O. niloticus* population reach sexual maturity early and start reproducing in grow-out ponds before they reach a marketable size, reducing the yield and value at harvest [20]. Therefore, commercial production of tilapia often relies on monosex culture of males. Beginning with the work of Hickling [15], a variety of methods have been used to produce monosex fingerlings, including interspecific hybridization [31], hormone treatment [23] and YY supermales [20]-[11]-[5]. These methods are not universally applicable**,** in part because of their technical complexity [30], but also because the sex of tilapia is affected by environmental factors such as temperature [1,3,8], and may also be influenced by autosomal genes [19]-[17]-[25].

Male mono-sex cultures are preferred to female ones, because of the differential growth in favour of males. While in females there is a greater reallocation of metabolic energy towards reproduction, in the males the metabolic energy is channelled towards growth. Additionally, males benefit from anabolism enhancing androgens [12].

Heat treatment as a means of producing mono sex Tilapia has been tried in several laboratories [1]-[4]. Sexual differentiation of gonads in Nile tilapia is triggered by temperature during the critical developmental period. Exposure to elevated temperature for 10 or more days between post fertilization days 9–13 increases the proportion of male individuals [3]-14].

Brain development is thought to have an influence on gonadal differentiation in tilapia. Sudhakumari [27] suggested that brain acts merely as a synchronizer in the sex differentiation process initiated by gonadal factors in the Nile tilapia. Brain aromatase and oestrogen receptors (ERs) are thought to be involved in brain differentiation in teleosts. Oestrogen-forming (aromatase) and oestrogen-sensitive (ER-containing) networks of neurones developing peri- and post-natally are crucial in brain differentiation [6]. Aromatase, a key enzyme for converting androgen to oestrogen [2], plays a role in neural differentiation and maturation in the brain and its activity and gene expression in neurones developing in the embryonic male brain is greater than in the female brain [16].

Although much is known about the process of sex differentiation in fish, the precise mechanisms involved in primary sex determination remain undefined [9]. Sex determination occurs through several mechanisms in teleosts. Primarily, sex determination has a genetic basis, which is

P. O. Angienda is with Maseno University, Department of Zoology, Maseno Kenya (corresponding author phone: +254 723701894; fax: ; e-mail: oyiengp@hotmail.com).

B. O. Aketch, is with Maseno University, Department of Zoology, Maseno Kenya (e-mail: akechben@yahoo.com).

E. N. Waindi is with Maseno University, Department of Zoology, Maseno Kenya (e-mail: enwaindi@yahoo.com).

determined at fertilization. This genetic mechanism usually occurs in one of two forms: heterogametic male (XY) or heterogametic female (WZ) [10]. Muller-Belecke & Horstgen-Schwark [21], however, reported that two or more sex determining factors might override the XX–XY mechanism in Nile tilapia. Therefore, knowledge of the relationships between sex-determining genes and sex steroids should help us to understand animal sex determination and sex differentiation in general, and to elucidate the conserved vertebrate sex-determining mechanisms. Recent studies on the genetics of sex determination in tilapiine species [7] have shown that sex determination in these species may be interplay between autosomal and sex linked locus. The objectives of the present study were therefore; to produce all male Nile tilapia by heat treatment technology as a method for producing fingerlings to local farmers to facilitate mono sex culture and to investigate the genetic basis of temperature induced sex determination in Nile tilapia.

II. Materials and Methods

A. The fish

A fresh stock of Nile tilapia obtained from little disturbed point of Lake Victoria was used for this study. The fish was trapped by use of seine nets and immediately transferred into aerated water in large plastic tanks. Only large sized tilapia, the lengths of an adult hand or longer, were retained in the tanks, while smaller ones were released back into the lake. The fish were transported in the aerated water tanks to our aquarium facilities, about 20 Km away. At the aquarium facilities the fish were sexed and those that were clearly determined as males or females, by visual examination of genital papillae, were transferred into separate concrete ponds designated as such, and let to acclimate for three months before commencing the experiments. They were fed on maize meal supplemented with 10% protein.

Out-door concrete water ponds of approximately $75M^3$ sizes, were used for breeding and brooding. These were layered at bottom with sand from the lake to mimic the natural environment. It had been observed that such a system is more conducive to natural spawning and fertilization of the eggs. The tanks were water aerated and replenished through the project water circulation system.

Sexually mature males and female, as determined by annul papillae and other physical and social characteristic, were transferred into the brood ponds at two females to one male, and let to brood and hatch freely. The hatched embryos at yolk sac stage were collected by scooping from the bottom of the tanks using plastic cups, pooled into one lot and transferred into indoor glass aquaria ($1.5M^3$). They were observed daily for yolk adsorption. One day after yolk adsorption constituted day one post yolk sac stage of development. Light feeding on juvenile feeds was also commenced at this time.

B. Heat treatment

Glass aquaria ($1.5M^3$) were used for heat treatments. These were fitted with constant temperature thermostat water heaters, aerator pumps, sand-fine gravel filters systems and mercury thermometers. The aquaria were filled with equal volumes of water and thermostats set at respective temperatures ranging from 34°C to 37°C. Treatments at lower temperatures (28 - 32°C) are not given here as the experimental results did not vary from the controls. The actual temperatures however, varied by ±0.5°C as shown by daily readings from the mercury thermometers. Temperature for control was 26°C. The temperatures were allowed to stabilize for several days before introducing the fry into the aquaria.

One hundred fry at 10 days post yolk sac were introduced into the aquaria for the various treatment temperatures Heat treatments were conducted for 10 days after which the thermostats were switched off and the aquaria allowed to cool down to room temperature; This normally takes about one day. During heat treatments the fry were observed daily and any deaths recorded. The final numbers of the fry in each aquarium were taken and recorded for at least 24 hrs post heat treatment. These were used to calculate survival rates. For further observations and experimental procedures, the fry at about 15 days post heat treatment were transferred to fry holding ponds where they continued to receive feed portions of 10g/kg body weight. At two monthly intervals, samples of about 10 fry were drown from each treatment pond in a small quantity weighed and returned into the ponds. The combined average weights were used to determine the feed portions. The stocking density was 3 fry/M^2 for all the ponds.

C. Sex ratios and weight measurements

At monthly intervals, up to 6 months post heat treatment, statistical samples of fry/fingerlings were drawn from the ponds for determination of weight measurements used for determination of growth rate for the different temperatures tested. The fingerlings drawn at 6 months post heat treatment were also used for sexing of the fingerlings. Sexing was done by microscopic examination of gonad quashes fixed and stained in aceto-orcein fix stain [28] and by fixing the gonads in Bouin's fixative, processing through standard histology method, and staining with eoisin-haematoxylin. Male individuals were identified by the presence of developing seminiferous tubules and spermatocyes, while females were identified by the presence of oocytes. The numbers of males and females were recorded for each temperature treatment. Sex ratios were calculated as percentages of the numbers of male or female fry sexed as such. Growth rates were determined from GSI (gonadal somatic index) at 60 days post heat treatment (dpht) and 120dpht. At 60dpht the gonads were still immature hence the need to obtain GSI again at 120dpht when they are fairly mature.

D. genotyping

Two sex linked microsatellite loci were selected from the Linkage group 9 of *Astotilapia burtoni*, homologous to linkage group 1 of Nile tilapia. The primers were Abur100 and Abur36 [26]. Nile tilapia sex linked locus, UNH846 [7],

from University of Hampshire was also included. The forward primers were labelled with a fluorescent dye (either 6-FAM, HEX or NED). PCR amplification was performed in a reaction volume of 20.0 µL, which comprises 1X PCR buffer, 25 µM of each dNTP, 0.5 µM of each of the forward and reverse primers, 0.1U *Taq* polymerase (Genaxxon) and 100-200 ng of DNA template, under the following thermal conditions: an initial denaturation phase at 94°C for 5 min followed by 35 cycles with a denaturation phase at 94 °C for 30 s, an annealing phase at 55 °C for 30 s, an extension phase at 72 °C for 90 s and a final extension phase at 72 °C for 10 min in Perkin Elmer GeneAmp PCR 9700 (Norwalk, CT). PCR products were diluted in formamide HiDi and electrophoresed in an ABI 3130xl automated sequencer. Fragment sizes were compared to ROX 500bp size standard (ABI) as determined using GENOTYPER® software (Applied Biosystems).

E. Survival rates

Survival rates were based on the numbers of fry that survived the heat treatment up to 24 hours post heat treatment and expressed as percentages of the initial numbers.

F. Statistical analysiss

STATA statistical analysis tool was used to obtain the correlation between the survival rates and treatment temperatures. Comparisons were made between inter-temperature sex shift and survival rates. The level for statistical significance was $P<0.05$. Correlation analyses between temperature and sex shifts and between temperature and survival were done at statistical significance of $P<0.01$. G test was used for analysis of the genetic proportions of sex linked markers in male and female individuals.

III. Results

Sexing was done by microscopic observation of the wet squashes of gonads fix-stained in aceto-orcein fix stain according to Waindi [28] and confirmed by histological method. Observation of the various stages of oogenesis development indicated female gonads, hence female (Fig. 1 a,b) while presence of seminiferous tubules and clusters of spermatocytes indicated male gonads hence male (Fig. 1 c,d).

The effects of heat treatments at various temperatures on sex ratios and survival rates indicated that there was a positive, but unproportional, correlation between treatment temperatures and the resultant sex ratios, while contrarily, treatment temperature and survival rates showed a negative correlation **(**Fig. 2**),** higher temperatures favouring sex shift towards males but being unfavourable to the survival of the fry. The maximum temperatures that provided for optimization of both sex shift (towards males) and the survival of the fry was found to lie within a narrow range of 36±0.5°C, providing the most optimal results of 86.31% males and 65.25% survival of the fry. Although higher temperatures gave higher male percentages, the survival rates were very low.

Fig. 3 shows results of differential weight gain presented as gonadal somatic indices (GSI). As seen in the figure, individuals treated at 36°C registered the highest growth rate. The 36°C treatment lot had the highest percentage of males. Males are known to grow faster and lager than females, hence the high growth rate.

The genetic proportions of sex linked genes are presented in Table 1. Abur36 predicted 95% phenotypic sex of the sex reversed individuals.

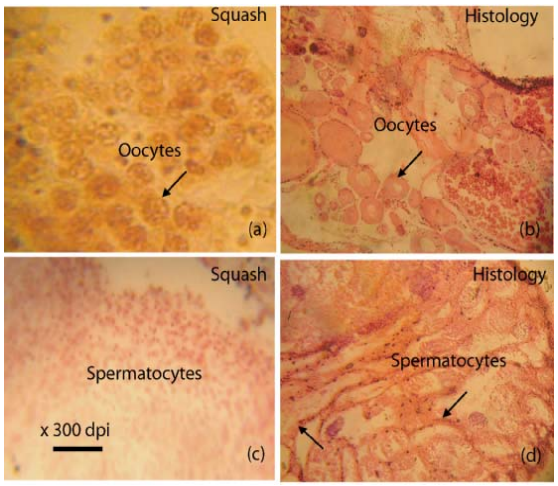

Fig. 1 Micrograph of squash (a) and histology preparations (b) showing the evidence of ovarian development with primary stage oocytes (a–arrows) and secondary stage (b–arrows) oocytes, and evidence of testis with spermatocytes and seminiferous tubules (d–arrows) and clusters of spermatocyes (c)

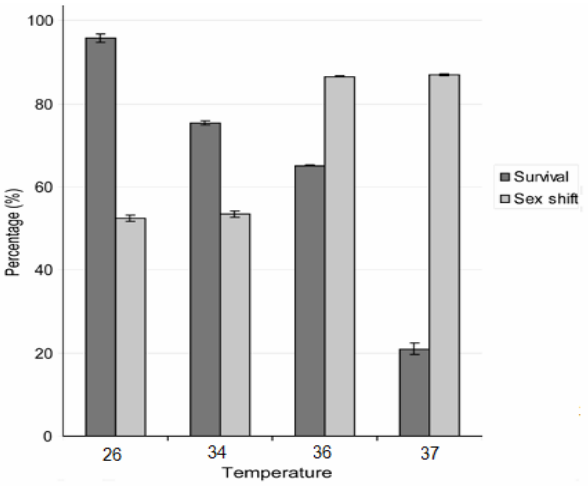

Fig. 2 Survival and sex shift (%) of the fry after heat treatments at indicated temperatures (ºC)

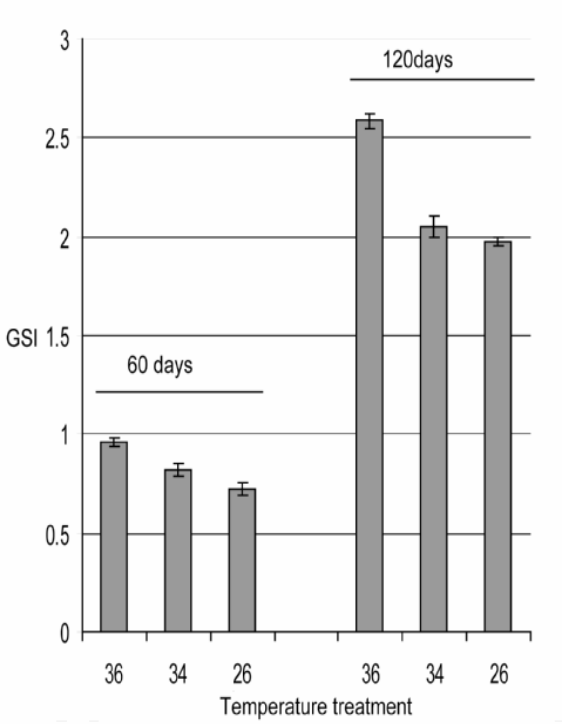

Fig. 3 Measure of growth of *Oreochromis niloticus* at 60 days post heat treatment (dpht) and 120dpht, showing the effects of heat treatment at the various temperatures, 26^0C being the control.

IV. DISCUSSION

The results show that treatment at 36°C gave most optimal conditions for both sex reversal (86.31%) and fry survival (65.25) (Fig. 2). At 37°C there was little change in sex reversal while there was a drastic fall in fry survival rate.. The observed optimal conditions for sex reversal may suggest the existence of a critical sex reversal temperature for Nile tilapia within a narrow range around 35.5°C-36.5°C. Such critical conditions have been observed for other fishes [13]. There is also a need to synchronise the ages at which the fry are heat treated and the treatment temperatures, as well as the length of treatment. *Oreochromis mossambicus* juveniles that were heat treated before 5 days of age showed a higher incidence of deformities than those that were equally treated but at older ages [29]. In our experiments heat treatments commenced 10 days post yolk sac stage (~ 18 days post hatch) and lasted only 10 days. No abnormalities were observed in the fry that survived the various treatment temperatures.

In the current and several previous studies [29,22,8], it has been shown that elevated temperatures favour masculinity in *O. niloticus* and other fishes. Thus the difference in growth rates in favour of sex reversed individuals. In the present study, sex could be unambiguously determined by squash mounts, the gonads were generally big enough for squash where the presence of developing oocytes and distinctive testicular structures could be observed in histological preparations (Fig. 1). In both methods, spermatogonial clusters and developing oocytes were observable indicating the accuracy of the squash technique. The oocytes appeared as small rounded cells with very high nucleus to cytoplasm ratio.

TABLE I
GENOTYPIC PROPORTION OF SEX LINKED MARKERS IN MALE AND FEMALE *OREOCHROMIS NILOTICUS*

		Control (GSD)			Sex-reversed (TSD)		
Maker	Genotype	M	F	G-test	M	F	G-test
Abur100	368/368	2	6	15.3	11	5	34.45**
	368/386	9	16		14	3	
Abur36	211/213	5	12	32.7**	0	1	68.23*
	211/227	11	0		21	2	
	227/227	6	1		9	0	
UNH846	179/209	2	7	20.15	4	6	23.11
	179/205	9	14		16	0	

*$P < 0.05$, **$P < 0.01$
GDS (Genetic sex determination), TDS (Temperature sex determination), M= males, F= females.

Our results also indicated that that raising and maintaining temperature at 36.5 ± 0.5^0C, has an effect on sex determination of *O. niloticus*. These results confirm that temperature is important in gonadal sex determination in tilapias as reported by Baroiller and D'Cotta [4]. This is in agreement with the observation that sexual differentiation of gonads is triggered by temperature during the critical developmental period and that exposure to elevated temperature for 10 or more days between post fertilization days 9–13 increases the proportion of male Nile tilapia [3]. and that gonadal sex is determined by temperature before the onset of gonadal differentiation in fish [14].

Sex-differentiation in fish is controlled ultimately by specific sex-determining genes, but genetic and phenotypic ratios do not necessarily coincide and interaction between the genome and variable environmental and internal factors may determine sex [18]. Previous studies have shown that sex determination in Nile tilapia occurs through several mechanisms, though primarily sex determination has a genetic basis, which is determined at fertilization, the genetic mechanism usually occur in one of two forms: heterogametic male (XY) or heterogametic female (WZ) [10]. However Muller-Belecke & Horstgen-Schwark [21] reported that two or more sex determining factors might override the XX–XY mechanism in Nile tilapia. Therefore we investigated the effect of temperature on sex linked genes and the resultant sex proportions. The genotypic proportions in males and females, and the associated G-tests, are shown in Table 1. These markers on linkage group 1 (LG1) showed significant differences in genotypic frequency between males and females The results indicated that the locus Abur 36 could be an 'autosomal locus' affecting sex ratio and its interaction with environmental factors e.g. temperature as shown in this study has a resultant male biased sex ratio. This locus could as well turn out to be the major sex determining gene operating in

Nile tilapia.

The mechanism of temperature dependent sex-determination in Nile tilapia is still not clear. However this is an attempt to predict the mechanism by investigation the effect of heat on the role of the sex linked genes. Previous studies have stipulated that temperature could activate at least four genes: Gene encoding for aromatase receptors and oestrogen receptors at female producing temperatures and genes encoding for 5α-5β reductase receptors and androgen receptors at male producing temperatures [24]. It may also act on the metabolic pathway for steroid biosynthesis or brain. However Sudhakumari [27] suggested that brain acts merely as a synchronizer in the sex differentiation process initiated by gonadal factors in the Nile tilapia.

These DNA markers have potential utility for tracking sex-linked haplotypes in breeding programs aimed at controlling the sex of fingerlings for commercial production. Marker-assisted selection could then be used to select genotypes that give a higher percentage of males for commercial production. Therefore the heat induced sex-determination mechanism in Nile tilapia can be modelled from this genetic approach. It is therefore clear that heat has an effect on the sex-linked genes and or autosomal genes with a resultant effect of a male biased cascade of events. Temperature dependent sex reversal requires obtaining recently hatched fry and rearing them in aquaria with high quality water. It has the potential as the most effective and user friendly in terms of production and adoptability to local farmers for seed production in facilitating mono sex culture of all-male Nile tilapia.

In the interest of Nile tilapia aquaculture development, the question that may arise from our results is whether the 13.69% of female fry in the 86.31% monosex male cultures would cause a serious over population problem. In the tropics tilapia fingerlings become sexually mature at about 4-5 months of age. They attain market sizes at 7-9 months, at which time the whole pond is harvested. This may allow the females to have only one cycle of reproduction. This situation therefore, may not present a serious overpopulation problem.

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