Effects of triploidy induction on growth and masculinization of red tilapia
[Oreochromis mossambicus (Peters, 1852) × Oreochromis niloticus (Linnaeus, 1758)]

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A B S T R A C T

The present study aims to evaluate the effects of triploidy induction by temperature-shocks (heat shock at 41 °C for 5 min and cold shock at 9 °C for 30 min of duration, starting 4 min after fertilization) on growth parameters and the sex ratios in progenies of three different red tilapia brood stocks under tank culture conditions for 120 days. A significant difference in total yield (P < 0.05) was recorded in favor of the heat-shocked induced triploid groups. Total average body weight of male fish in all the groups showed more weight gain than the females, during the culture period (P < 0.01). Statistical analysis of the total weight gain among the sexes revealed that there was no significant difference between the average weights of male belonging to various groups, whereas in females the heat-shocked group showed a significant increase in weight compared to that of diploid females (P < 0.05). Red tilapia subjected to heat-shock treatment showed positive correlation (P < 0.001) with sex ratio, where skewness towards male progenies (84.1%) was observed as compared to cold-shocked and control groups. Highly significant difference in ovary weight and GSI was assessed between triploidy induced females and diploid females of the control group (P < 0.001). The impact of heat shock and cold shock induction of triploidy on the performance and masculinization is discussed in detail.

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1. Introduction

The curiosity behind the sex determination mechanism in tilapia is motivated by the practical and commercial implications in the production of monosex male populations for aquaculture (Desprez et al., 2006). The sex differentiation into male or female is a complex and labile mechanism under genetic control including a mixture of major (XX/XY and ZZ/WZ) and minor factors (autosomal) (Baroiller et al., 1999, 2009; Desprez et al., 2003; Nagahama et al., 2004; Rubin, 1985; Sullivan and Schultz, 1986; Tessema et al., 2006). Most of the work on environmental sex determination in tilapia has focused on the effects of temperature. Earlier studies on various tilapia species have shown that high-temperature treatments during the early developmental stage (labile period) cause a significant skewness of sex ratio in favor of males (Baras et al., 2000, 2001; Baroller et al., 1995, 1996a, 1996b; Desprez and Me’lard, 1998) while lower temperature treatments resulted in skewness towards female (Baroller et al., 1996b). These findings suggest that an early sex differentiation pathway during the embryonic stage is prevailing in tilapia (Rougeot et al., 2008). However, there is a paucity of information regarding the various environmental effects on sex differentiation during the embryonic development before hatching (Rosenstein and Hulata, 1992).

Production of sterile tilapia through triploidy induction has attracted considerable interest in the past (Mair, 1993). All the earlier studies have reported that the high levels of triploidy can be achieved in tilapia using various shock treatments soon after the fertilization (Chang and Liao, 1996; Chourrout and Itskovich, 1983; Don and Avtalion, 1986, 1988; Hussain et al., 1991; Penman et al., 1987; Valenti, 1975; Varadaraj and Pandian, 1988). Production of all-female sterile triploid tilapia has been attempted by Varadaraj and Pandian (1990) and they have suggested that all-female-sterile tilapia may have considerable potential for tilapia aquaculture. In fact, production of sterile triploid males can also be considered as a holistic approach to an alternative method for monosex technique. All-male tilapia populations are a desirable solution to control the prolific...
breeding activity. Moreover this solution improves the yield in tilapia aquaculture, since the male grows faster than the female in this species (Macintosh and Little, 1995; Myers et al., 1995; Tessema et al., 2006). Furthermore, application of monosex in tilapia aquaculture would offer several other advantages like; reduction of sexual/territorial behavior, reduction of variation in harvest size, and reduction of risk of environmental impact resulting from escape of exotic species (Beardmore et al., 2001).

Previous studies on triploidy induction in tilapia, has shown to select female sex (Byamungu et al., 2001; Mol et al., 1994). However, no reports have ever been made on the influence of temperature shocks during triploidy induction in altering the sex ratios of tilapia in favor of males. Since temperature shocks are applied soon after the fertilization, possibility of modifying the sex ratios right from the time of fertilization may be possible. Hence, comprehensive experiments were carried out with the aim to evaluate the effects of temperature shock triploidy induction on sex ratios and growth in red tilapia.

2. Materials and methods

2.1. Origin of fish and brood stock management

The experimental fish used in this study were commercial hybrid red tilapia strain commonly known as red tilapia, Oreochromis mossambicus (Peters, 1852) × Oreochromis niloticus (Linnaeus, 1758). Fresh stocks of red tilapia adult males and females used in the present study were obtained from a cage culture farm located in the Lake Kenyir, Terengganu in Malaysia. This study was carried out at the Freshwater Aquaculture Unit of University of Malaysia in Terengganu. Brooders were fed twice a day with pelleted feed (43% protein, ASEAN Marine Fish Feed, Ltd.). In addition to ASEAN Marine Feed, fish were also given ad-libitum with Cabomba caroliniana once in a week to satisfy their vegetative requirements. The fish were maintained under natural conditions of photoperiod and temperature (26–27 °C) in 5000 l rectangular cement tanks with proper aeration. A defined water quality were kept in the tank with oxygen > 7 mg/l; pH 6.5–7.0; NH4+ <0.25 mg/l; and NO3− <0.2 mg/l.

2.2. Spawning

Before starting the experiments, the brooders were starved for 24 h. Three pairs of brooders [males (average weight: 223.7 g) and females (average weight: 236.4 g)] were selected from the cement tanks on the basis of readiness for spawning as suggested by Rothbard and Pruginin (1975). For better ovulation and spermiation, the selected brooders were induced by HCG (Pregnyl1500) injection (1500 IAU/kg body weight) which was applied just below the dorsal fin. Each pair of fish was kept in 120 l-aquarium tank. The fish were kept separated by a sheet of Perspex. The aquarium tank was provided with constant water temperature (28 ± 1 °C) controlled by a digital heater (Model-D-38300, 300 W-Italy) and adequate aeration. Ovulation and spawning readiness during the experiment time were determined by observation of courtship behavior, coloration and papilla erection (Rothbard and Pruginin, 1975).

2.3. Gamete collection

Females were stripped after the initiation of spawning, i.e. after the release of the first batch of eggs (~20–30 nos.). Simultaneously, the partner male was also stripped. Soon after the gamete collection, the eggs were fertilized with 0.6–1 ml of milt diluted with a small quantity of freshwater (28 ± 1 °C). After a minute, the eggs were rinsed with fresh water to avoid polyspermy and then divided into control and experimental batches.

2.4. Triploidy induction

From each brood stock pair (N = 3), in vitro fertilized eggs were collected and divided into three equal batches consisting of 350–400 eggs/batch. The shock treatment on fertilized eggs collected from each pair was considered as replicates. Two batches were used to produce triploids (3N) by heat (41 °C for 5 min duration, 4 min after fertilization) Pradeep et al. (2010) and cold (9 °C for 30 min duration, 4 min after fertilization) shocks (Pradeep, 2011). The third batch was untreated and was considered as a normal diploid control (2N).

2.5. Egg incubation and calculation of survival

After the shock induction, eggs were counted and transferred to the incubation chamber along with control group for further development. All fertilized eggs of both treated and control groups were incubated identically in round bottomed glass jars (250 ml) connected to a recirculatory incubation system (Pradeep et al., 2011a). The fertilized eggs were counted at the blastula stage i.e. 10 h after the fertilization (a.f.) and hatching rate at 80–90 h a.f. The survival rates were taken on the 5th day (120 h a.f.) and 120th day, during the termination time of the experiment.

2.6. Ploidy evaluation

Ploidy evaluation was performed by chromosome preparations from one day old red tilapia larvae. Chromosome counts were made on a sub-sample (N = 10) from each batch (Pradeep et al., 2011b). Further verification of the ploidy was done when the fish had reached 60 days of age using mean cellular volume of the erythrocytes (Pradeep et al., 2011c). The blood samples of each fish from all treatments were collected and verified for ploidy, without killing the fish.

2.7. Fry and fingerling rearing

After hatching, 220 larvae (0.011 g/larva) were removed from each group and stocked separately in nine glass aquarium tanks (550 l). The temperatures of the aquarium tanks were maintained at 28 ± 1 °C using digital heater (Model-D-38300, 300 W-Italy) until they were transferred to cement tanks (age 30 days) for further development. Initially, larvae were fed with newly hatched Artemia nauplii at a rate of 10–15 individuals/ml, 3–5 times a day for a week. This was followed by feeding the fries with powdered feed (ASEAN Marine Fish Feed with 43% protein) at a ratio of 12–15% of their body weight, thrice a day for a period of 10 days. After the 30th day, fries (N = 180) were transferred to cement tanks (3.5 × 2 × 2 m) where the level of water was constantly maintained at 1.5 m throughout the experimental period. The feeding rate was subsequently reduced to 8–10% of the total biomass allowing fishes to feed 2–4 times a day. The feeding program was re-scheduled after the 40th day and there after the fish was fed twice with commercially available sea bass pelleted feed at 5% of their body weight. The daily ration was calculated every 15 days and for that purpose the average body weight of 20 fish in each tank was taken and accordingly rationing was determined using the following equation:

\[
\text{Daily food ration (DFR)} = \frac{\text{Average body weight} \times \text{No. of Individuals} \times \% \text{ food requirement}}{100}
\]

2.8. Water quality management

All the nine batches (3 treatments with their replicates) were maintained under similar culture condition in the cement tanks to avoid any experimental error. Water quality was maintained throughout the
experimental period by daily exchange of 40% water. Monthly cleaning was done in the cement culture tanks to remove algal growth and other decayed organic waste materials. The quality of water was also monitored weekly with the help of a master test kit (Aquarium Pharmaceuticals, INC) where nitrite (NO$_2^-$), pH and ammonia were approximately kept at <0.2 mg/l, >7 mg/l and 0.25 mg/l, respectively. Mortality, if any was recorded and accordingly feeding ration was altered to maintain a uniform feed quantity throughout the culture period.

2.9. Estimation of final weight and sex ratio

The fish were starved on 120th day; their final weight was recorded accordingly. Since poor development of the triploid gonads made it difficult their visual sex identification, the fish were dissected individually for their accurate sex determination. However, before sacrificing, the individual weight of each fish (N = 120) from all the groups was taken using a single pan electronic balance (precision 0.01 g), while total length of each fish was calculated using a measuring scale. Total yield from each tank was calculated by adding all the recorded weights of 120 fishes. The other growth parameters like gonadal weight and gonadosomatic index (GSI) were also calculated where gonadosomatic index was deduced according to the formula as suggested by Razak et al. (1999).

Gonadosomatic index (GSI) = \frac{\text{Weight of gonad (g)}}{\text{Weight of fish (g)}} \times 100

In order to assess the stoutness of the fish, condition factor was calculated according to the formula as suggested by Razak et al. (1999). The study of condition is based on the analysis of length–weight data and assumes that heavier fish of a given length are in better condition (Bolger and Connolly, 1989).

\text{Condition factor (K) =} \frac{W}{L^3} \times 100

where W is the weight and L is the total length of the fish.

2.10. Statistical analysis

Standard statistical procedures were used for data processing using the statistical package SPSS, 16.0 and data were expressed as a mean ± SD. Comparison of the erythrocyte size, fish weight and length between diploids and triploids was made using one-way ANOVA, followed by Student’s t-test. Levene’s test was used to determine the homogeneity of variances. The data for the factors GSI, condition factor and gonad weight among diploid and ploidy groups were analyzed using one-way ANOVA. The influence of ploidy on sex ratio was verified using one-way ANOVA and goodness-of-fit to a 1:1 sex-ratio in each ploidy group was tested using Chi squared test ($\chi^2$). Two-way analysis of variance was performed for the survival rate, considering the two ploidy types — diploid and triploid, as fixed factors and the various development stages, as random effects. Differences were considered significant when P < 0.05.

3. Results

Ploidy analysis done using chromosome preparations from one day old larvae showed a triploid percentage of 94.4% in heat shock and 98.8% using cold shock groups. In control groups, all the fishes were diploid. Ploidy verification using nuclear major axis of erythrocytes at the age of 60 days revealed that the shape of the triploid erythrocytes was oval whereas it was almost round in diploid individuals. The means (±S.D.) of nucleus major axis were; 4.47 ± 0.34 and 5.89 ± 0.38 for diploids and triploids, respectively. This indicated that the mean length of triploid nucleus major axis was greater than those of diploids

\[ \text{Weight of fish (g)} = \frac{W}{L^3} \times 100 \]

\[ \text{Survival rate} = \frac{\text{Weight (g)}}{\text{Weight of fish (g)}} \times 100 \]

\[ \text{GSI} = \frac{\text{Weight of gonad (g)}}{\text{Weight of fish (g)}} \times 100 \]

\[ \text{Condition factor (K) =} \frac{W}{L^3} \times 100 \]

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Cold shock</th>
<th>Heat shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>210.1 ± 7.4a</td>
<td>215.1 ± 6.2a</td>
<td>223.4 ± 10.5a</td>
</tr>
<tr>
<td>Total length (cm)</td>
<td>17.7 ± 0.23a</td>
<td>17.8 ± 0.39a</td>
<td>17.7 ± 0.60a</td>
</tr>
<tr>
<td>Gonad weight (g)</td>
<td>1.91 ± 0.07a</td>
<td>1.85 ± 0.03a</td>
<td>1.85 ± 0.05a</td>
</tr>
<tr>
<td>GSI</td>
<td>0.93 ± 0.02a</td>
<td>0.87 ± 0.04a</td>
<td>0.84 ± 0.04b</td>
</tr>
<tr>
<td>Condition factor</td>
<td>3.9 ± 0.2a</td>
<td>3.9 ± 0.3a</td>
<td>4.1 ± 0.5a</td>
</tr>
</tbody>
</table>

\[ \text{Mean (±SE), a common superscript in rows indicates values which do not differ significantly (P > 0.05); superscriptsa,b indicate that they are not significant in both groups.} \]

Fig. 1. Mean survival rate of diploid and triploid embryos at three developmental stages; blastula stage (10 h a.f.), hatching stage (80-90 a.f.) and yolk sac (120 h a.f.) (scale bar ± SE).
with both the control and heat-shocked groups. When the yield from each tank was compared, a significant correlation in growth enhancement in terms of weight was observed in favor of heat-shocked group (209.5 ± 5.9 g) in comparison to cold shock (184 ± 6.3 g) and control (177 ± 1.9 g) and level of significance was at P < 0.05 level. Though the total length was significantly different between the sexes in three groups, however within the groups there was no difference.

Testes of diploids were elongated, soft, milky and showed motile spermatozoa, whereas spermatozoa of triploids was somewhat watery. Triploid testes were almost of similar in size as of diploid, but thin and flat (Fig. 2). No significant difference in the weight of testes was found between triploids of cold-shocked and heat-shocked groups as compared to diploid (control) males. The only significance was in the GSI of heat-shocked group with that of control counterpart (P < 0.05). High significant differences (P < 0.001) in ovary weight and GSI were found between triploid and diploid females at 120th day (Table 1). The ovaries of diploid controls were 16–19 times heavier than the ovaries of their triploid counterpart. The ovaries of the diploid females showed numerous developing oocytes, while the ovaries of the triploid females were very thin, string-like and occasionally short and plump (Fig. 2). Condition factor of both sexes in all the shock treated and controls did not show much variation (Table 1).

The mean survival rates in the temperature-shocked groups were significantly lower than those observed in the control groups during the termination of the experiment (Fig. 3). An interesting bias in the sex ratio was observed in the present study. A high significant correlation was found between the sex ratios and the shock treatments. The fish subjected to heat-shock treatments at 41 °C showed skewness towards males with respect to both the control (28 °C) and the cold-shocked treatments (9 °C) (Fig. 4). A maximum of 84.1% of male individuals were recorded in triploid fish produced through heat-shock, whereas 54.7% of males were present in the cold-shock stock, followed by the control with only 50.9% of males.

4. Discussion

In this study, triploidization was done successfully, which yielded a percentage of 94.4 triploids by heat shock and 98.8 by cold shock in red tilapia. At the time of the experiment termination at 120 days, all triploid tilapias of both sexes were heavier than their diploid siblings (Table 1). Valenti (1975) also reported that the triploid blue tilapias were heavier than their diploid counterparts. But growth performances of triploid tilapias have always given contradictory information so far. Several researchers have suggested inferior or similar growth performance for triploid tilapia. In fact, all these experimental results were reported under laboratory conditions (Don and Avtalion, 1986; Hussain et al., 1995; Penman et al., 1987; Puckhaber and Horstgen-Schwark, 1996). The presence of higher female percentage together with lower growth rate of female tilapia has also been found to influence the reduced growth performance in some triploid tilapia species (Byamungu et al., 2001; Mol et al., 1994). However, triploid tilapia culture under farm condition for longer period or even manipulation of feeding regimes have shown to influence growth in favor of the triploid tilapia (Bramick et al., 1995). Hence, it is necessary for comparing the growth performance of diploid and triploid tilapia, we have to consider two different growth phases, before and after sexual maturity. This is because triploids did not exceed the growth of the control fish stock up to sexual maturity as observed from early studies (Mair, 1993).

The present study showed a significant difference (P < 0.05) in average body weight between the males and females for all treatments. Heat-shocked males showed the maximum average body weight difference among sexes of all the groups tested, but the difference was not statistically significant with respect to the other male groups. However,
average body weight of heat-shocked females showed significant higher values (P<0.05) as compared to diploid female but not with respect to the cold-shocked female. This was in agreement with other studies in which triploid males showed higher body weight than their female siblings (Bramick et al., 1995; Hussain et al., 1995, 1996; Mol et al., 1994). In most of tilapia species, growth increment in males is probably due to their digestive efficiency and higher capability of nutrient metabolism (Toguyeni et al., 1996). Another probability for the growth advantage in males is due to the involvement of the hormonal status by effect of steroid (11-keto-testosterone) and thyroid hormone (3,3′,5-triiodothyronine) levels on nutrient metabolism (Byamungu et al., 2001; Mol et al., 1994; Toguyeni et al., 1996). In addition, the growth enhancement in triploid tilapias might be due to the effect of sterility, a condition that may divert most of the energy and nutrients for somatic growth rather than for the gonadal development and sexual activity.

In our study the most desirable and striking difference among the various traits of sexes were the gonad weight and the GSI in the triploid females, compared to the diploid control. Triploid females always showed small and underdeveloped ovaries, which indicates that sterility through triploidy induction has arrested the ovarian development. However, there was no significant difference on the weight of male testes between the triploids and diploids. Only significance was seen in the GSI of heat-shocked group with that of the control counterpart. Other researchers also reported delay of the gonadal development in triploid tilapia upon maturation as compared to diploid siblings (Hussain et al., 1995, 1996; Pandian and Varadaraj, 1988, Penman et al., 1987; Puckhaber and Horstgen-Schwark, 1996; Varadaraj and Pandian, 1990). All these studies have suggested that in triploids of both sexes, average gonadal development, as well as relative weight of gonads (expressed as GSI) were comparable to those found in diploid counterparts. A few studies have reported unexpected high GSI and ovarian appearance in triploids similar to that of diploids under field condition (Bramick et al., 1995; Penman et al., 1987), which was contradictory with respect to the results obtained in laboratory conditions. It has been reported by Bramick et al. (1995) that triploid females with low GSI displayed significantly higher body weight than the females with high GSI. This indicates a negative impact of advanced gonadal development on growth rates in triploid females.

Our results prove the possibility of skewing the red tilapia into phenotypic males using heat-shock induction of triploidy. Comparatively higher percentage of males (84.1%) observed in heat-shocked triploids resulted in higher yield (209.5±5.9 g), when the average weight of each group was calculated. Previously, Mol et al. (1994) suggested that the induction of triploidy showed no significant effect on the growth of females and males individually, but it altered the sex ratio and therefore the growth rate of the whole population. The findings from Mol et al. (1994) was true in our studies, where we have observed that heat-shock induction of triploidy was capable in altering the sex ratio and thereby enhancing the yield of whole population. In fact, it has been proved that the earlier study of Bramick et al. (1995) longer culture period in pond condition has significant effect on growth of triploid tilapia in both the sexes that were produced by heat shock. Their study further showed that, though no significant difference in growth performance was observed between triploids and diploid of O. niloticus, until the age of maturation, however, triploids were significantly heavier (P<0.01) than diploids at the end of the 285 days of the experiment. Moreover their results showed a decreased sex dimorphism in triploid tilapia. In their study at the time of termination, the diploid males displayed 25±5.9% higher body weights than diploid females, but growth advantage of triploid males was still significant (P<0.01) but reduced to average values of only 8±3.4% at the final drainage. These results suggested that discarding of females because of the inferior growth rates, which is commonly practiced in traditional tilapia culture systems, would become needless in triploid populations. Hence it is anticipated that similar trend as reported by Bramick et al. (1995) could have been achieved in the present study with red tilapia, if the culture period would have continued for some more days.

Several researchers have reported that the triploidisation of various tilapia species resulted in a higher proportion of females (Byamungu et al., 2001; Mol et al., 1994; Pandian and Varadaraj, 1987; Penman et al., 1987). However, studies by Chang et al. (1991) in blue tilapia and Hussain et al. (1995) in Nile tilapia, did not show any difference of sex ratio between diploids and triploids. Since, both parent species (O. mossambicus and O. niloticus) were not showing any difference in sex ratio as revealed from the previous studies, we did not consider their effect on triploidization and sex ratio in our study, but only on their hybrid strain. Recent reports suggest that the process of sexual differentiation in tilapia takes place in the brain before than in the gonad (Arnold, 2004; Matsuoka et al., 2006; Sudhakumari et al., 2005). The possible mechanism behind the influence of temperature on the sex differentiation process during embryogenesis is not yet understood. A reasonable “brain sexualisation” hypothesis has been suggested by many researchers. The high temperature could directly act on the brain aromatase gene (CYP19B) modifying the sex differentiation pathway during embryogenesis (Morrison et al., 2001). The aromatase gene (CYP19B) produces aromatase cytochrome P450 which is a terminal enzyme in the estrogen biosynthetic pathway that catalyzes the formation of estrogen from androgen (Chang et al., 2005). The application of high temperatures at early embryonic stages have revealed to down regulate the expression of these genes or transcriptional factors responsible for the expression of the aromatase gene during sexual differentiation in fish (Tsai et al., 2003). One another hypothesis regarding the temperature influence on the early sexual differentiation is that, it could act directly on the future gonadal cells either somatic and/or germinal germ cells (Morrison et al., 2001; Rougeot et al., 2008). Although both these hypotheses shall be applicable for explaining the action of skewness in red tilapia using heat-shock induction, further investigation becomes a necessity for uncovering the mechanism behind this abnormal sex ratio.

The present research thus has proven that the use of sterile triploid tilapia may have future perspective in aquaculture and may be helpful to negotiate the problems associated with precocious maturity and excessive reproduction in tilapia. However, the technical constraints of artificial breeding along with laborious and time consuming practice for large scale triploid production has restricted the applicability of this technique to a limit in the past. A concerted effort is thus primarily be required to reduce these aforementioned constraints for the large scale applicability of triploidy induction in tilapia.

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Fig. 4. Effect of temperature shock on male sex ratio of diploid and triploid individuals (heat and cold-shocked) (scale bar ± SE).
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