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Temperature Incubation During the Embryo-Larval Stage for Inducing Sex Reversal of Nile Tilapia Red NIFI Strain

Mohamad Soleh[1], Rahma Aulia[1*], Agustien Naryaningsih[1], Lisa Ruliaty[1], Abidin Nur[1]

[1]Research Center for Fishery, National Research and Innovation Agency, Bogor, Indonesia.

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Corresponding Author:
Rahma Aulia
rahma.aulia@brin.go.id

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Abstract: This research aims to achieve male sex reversal in Nile Tilapia (*Orechromis niloticus*) of the red NIFI strain through high-temperature incubation during the embryonic or pre-swimming larval phase. Larvae resulting from the natural mating of red NIFI strain broodstock were incubated under temperature treatments of 31°C, 33°C, 35°C, and a control group (<30°C), each with three replicates. Temperature regulation was facilitated using a heater. During the initial 3 hours of incubation, the heater was turned off for 6 hours, followed by another 3 hours of incubation with the heater on. This pattern was repeated daily until the larvae began to swim. The treated larvae were then reared for 90 days. Thirty specimens from each replicate were identified for gender using the squash acetocarmine method. The study results indicated that temperature differences significantly influenced ($p<0.05$) the sex ratio of Nile tilapia in the red NIFI strain. The highest proportion of males was achieved at 33°C, at 73.33%, and 35°C, at 64.43%. There were no significant differences ($p>0.05$) in fry survival rates at 7 days and after 90 days of rearing.

Keywords: larva; masculinization; temperature; tilapia; sex reversal

Introduction

Nile tilapia (*Oreochromis* sp.) is one of Indonesia's flagship export products (Suseno et al., 2020). Its aquaculture development mainly takes place in freshwater ponds, and now there is a growing trend of cultivation in brackish water ponds (salted tilapia). The demand for Nile tilapia fingerlings in aquaculture is substantial and requires a mix of both male and female genders. In Nile tilapia aquaculture, a phenomenon exists where male fish tend to grow faster than their female counterparts. Female tilapia matures too quickly or experience early gonadal maturation (Sipayung et al., 2010). Male Nile tilapia exhibit faster growth rates and larger body sizes compared to females (Bardhan et al., 2021; Kembenya & Ondiba, 2021). Cultivating male Nile tilapia alone is known as monosex tilapia farming (Suseno et al., 2020). To meet the demand for monosex culture, one approach is to provide all-male fingerlings through the process of sex reversal. Sex reversal is a form of environmental sex determination (ESD) used to produce the opposite sex through environmental manipulation (Renn & Hurd, 2021). Hormonal methods are often employed in monosex fingerling production but may have side effects.

In general, sex reversal is a physiological, not genetic, process, meaning that the change in sex (gender) is purely physiological (gamete production, sperm, or oocytes) without altering the fish's genetics. Fish with female genetics can physiologically change into males and produce sperm (masculinization), while fish with male genetics can physiologically transform into females and produce oocytes or eggs (feminization) (Suseno et al., 2020). This process involves shortcuts, directly switching off one gender to the opposite gender, either by inhibiting the primary gender or inducing the phenotype of the opposite sex. Therefore, sex reversal implies that the phenotypic gender is contrary to the genetic or chromosomal gender. This process occurs during embryonic development or before gonadal differentiation (Weber & Capel, 2018). The differentiation into male or female functions is a

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complex and labile mechanism under the control of genetic, physiological, and environmental factors (Baroiller et al., 1995; Devlin & Nagahama, 2002).

Non-hormonal methods for sex reversal in fish include genetic manipulation, hybridization, or treatments affecting sex determination or gonadal differentiation through factors such as temperature, population density, pH, and other social factors (Weber & Capel, 2018), as well as physical treatments (pressure and water temperature) and chemical substances (Suseno et al., 2020). High water temperatures in the aquatic environment are considered safe for producing male fish (Baras et al., 2001; Bardhan et al., 2021; Baroiller et al., 1995; Tessema et al., 2006). The employed temperature treatment starting from the hatching of fish eggs and before fish start swimming (yolk sac stage) (Jun, 2021). Controlling water temperature within the range of 30-37°C resulted in 80-90% males, with an optimal water temperature of around 35°C.

Sex reversal in Nile tilapia using temperature treatment is carried out during the embryonic or pre-swimming larval phase, while still with the yolk sac. Temperature control is done cyclically (thermocycle) because this method is more effective in the sex differentiation process compared to constant temperature control (de Alba et al., 2023). The optimal frequency and duration of temperature treatment each day can vary. The research aims to achieve optimal male sex reversal in Nile tilapia by high-temperature incubation during the embryonic or pre-swimming larval phase to obtain the desired male sex ratio.

Method

Temperature treatment

The embryos or larvae were reared in a freshwater medium with a volume of 50 liters and incubated at their respective temperatures. The temperature treatments for water were 35°C, 33°C, and 31°C, controlled using heaters, and a control group (<30°C) without a heater, each with three replicates. Each plastic box was equipped with a 75-watt metal heater with a maximum temperature of 35°C. The incubation process started with three hours of heating, followed by a six-hour heater-off period, then another three hours of heating, and a subsequent 12-hour period without heating until the next day. The temperature treatments continued until the larvae began active swimming (without yolk sacs), and their populations were counted.

Larva rearing

The actively swimming larvae were maintained in a 50 L freshwater medium indoors. Larvae stocking density was adjusted based on the yield per female parent. The larvae were reared with water temperature controlled at approximately 30°C for three months (90 days), and the population of fish fry was counted monthly. The fish were fed commercial pellet feed in powder and fine-grain form, with a protein content of around 34%. Feeding was done with a feeding regimen of 20-15% (30 days I), 15-10% (30 days II), and 10-7% (30 days III) of fish biomass, for fish weighing 0.02, 1.0, and 5 grams, respectively (DeLong et al., 2009). Feeding was conducted twice daily. Water replacement in the rearing tanks was approximately 50% of the water volume, performed twice every seven days.

Determination of sex proportions

The determination of the male and female sex ratio was performed through chemical analysis using the acetocarmine squash sexing method (Guerrero & Shelton, 1974). A random sample of 30 fish fry with weights ranging from 0.17 to 3.73 g and lengths from 2.1 to 5.5 cm, each aged 3 months, was taken from each treatment replication. These fish were then prepared using a lethal dose of anesthesia (2-phenoxyethanol). The fish were dissected, and their gonads were removed, placed on a glass slide, and minced finely. The minced gonads were treated with 1-2 drops of acetocarmine, followed by air-drying until the gonad cells were stained with acetocarmine. The preparations were covered with a cover glass and examined under a microscope at 20x magnification. Female gonads were identified by the presence of large, round oocyte precursor cells, while male gonads were identified by the presence of small, dot-like spermatocyte precursor cells (Bhagawati et al., 2017). Observations were conducted at the Development Center of Brackishwater Aquaculture in Jepara.

Statistical analysis

The influence of treatments on the percentage of males, survival rate of one-week-old larvae, and three-month-old larvae was analyzed using the analysis of variance method (SPSS version 25) at a 95% confidence level ($p<0.05$). If there were significant differences among the treatments, a post hoc test, such as the Least Significant Difference (LSD) test, was performed. The data from each treatment were presented as mean values and standard deviations.

Result and Discussion

Results

A total of 360 fry were analyzed, with weights ranging from 0.17 to 3.73 g and lengths from 2.1 to 5.5 cm. The analysis results showed a significant difference ($p<0.05$) in the proportion of males, with 73.33% at 33°C

and 64.43% at 35°C compared to 31.00% at 31°C and 39.62% in the control group (<30°C) (Figure 1).

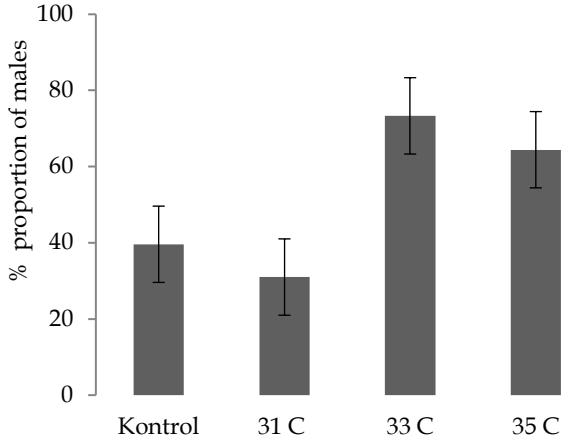

Figure 1. Proportion of male tilapia seeds aged 90 days.

Seven days after the temperature and control treatments, the survival rate of fry ranged from 84.89% to 95.05% (Figure 2) and showed no significant differences. The lowest fry survival rate was 84.89% at 35°C, while the highest was 95.05% at <30°C. The survival rate of fry after 90 days for all treatments ranged from 18.34% to 29.97% (Figure 3) and did not show significant differences. The highest survival rate of fry was 29.97% in the <30°C treatment, while the lowest was 18.34% at 35°C. Water quality parameters, including dissolved oxygen, temperature, and pH, during the 90-day rearing period were within the ranges of 5.1-6.4 ppm, 27.0-30°C, and 7.2-8.0, respectively. For the control treatment, the values were 5.5-6.8 ppm, 26.2-28.2°C, and 7.1-7.9, respectively. Overall, these parameters remained within the normal range for fry rearing.

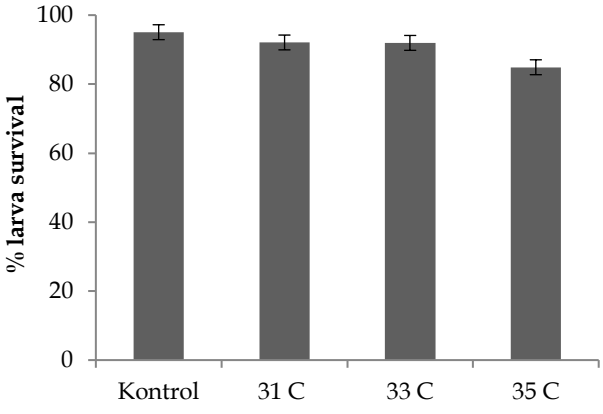

Figure 2. Larva survival seven days after treatment.

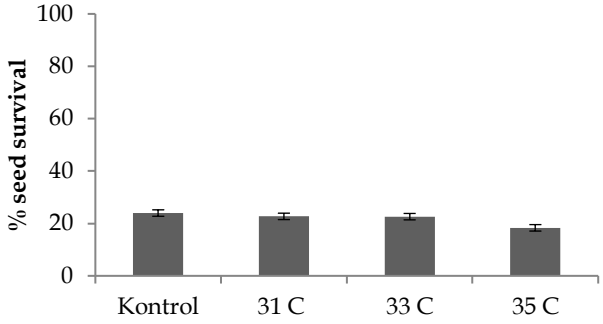

Figure 3. Tilapia seed survival, 90 days after treatment.

Discussion

The highest proportion of males was produced in the 33°C treatment at 73.33%, still lower compared to the previous research (Jun, 2021). Several factors may have influenced this, including the accuracy of the frequency and timing of temperature incubation, gradual fry mortality until day 90, and the possibility of individual fish changing their function to become males. The cumulative active heating time for each day was 6 hours, with 18 hours of heating cessation over a seven-day period. Temperature treatments not applied immediately after fertilization or with short temperature incubation times can result in different masculinization rates (Rougeot et al., 2008). Significant masculinization in *O. mossambicus* was achieved with a temperature treatment of 32°C compared to 20°C, applied for 5 days starting from newly hatched eggs (Wang & Tsai, 2000). The previous research conducted temperature treatments ranging from 30-37°C starting from the moment the fish eggs hatched and before the fish fry started swimming (Jun, 2021). They obtained more than 90% male individuals at the optimal temperature of around 35°C. Maximum proportion of male *O. niloticus* was achieved from Lake Manzala-Belgium strain at a temperature of around 36°C, with up to 26.7% (Rougeot et al., 2008). Complete masculinization was achieved at 36°C, but with the addition of 17α-methyltestosterone hormone (Sarker et al., 2023; Wang et al., 2022). Egg incubation and hatching time of 51 hours may allow for the change of female genotype into a male phenotype. A temperature of 27°C identified 100% female genotype (Baras et al., 2001; Baroiller et al., 1995; Tessema et al., 2006). It is important to note that the efficiency of sex reversal can vary among progeny and may be influenced by parental or maternal effects. Male fish tend to be more prevalent at higher temperatures (Ospina-Álvarez & Piferrer, 2008; Renn & Hurd, 2021).

During the sex differentiation stage at temperatures between 28°C to 36°C, the growth of female Nile tilapia that have undergone masculinization was proven to be faster, although their ovaries also developed (Habibah et al., 2021). This masculinization occurs due to changes in

mRNA expression (Lu et al., 2022). High temperatures are associated with the regulation of aromatase gene expression during fish sex differentiation (Baroiller & D'Cotta, 2001; D'cotta et al., 2001; Tsai et al., 2003), and sex differentiation primarily takes place in the brain before affecting the gonads (Arnold, 2004). The brain aromatase gene directly influences sex differentiation during embryogenesis. There is a hypothesis that sex determination in Nile tilapia is governed by the brain, occurring around 31 hours after fertilization and within a temperature range of 27°C to 29°C (Morrison et al., 2001). In Nile tilapia, the initial expression of the aromatase gene begins between day 3 and day 4 after fertilization (Kwon et al., 2001). The expression of brain aromatase and estrogen receptor in *O. mossambicus* is regulated by temperature and developmental periods during sex differentiation (Tsai et al., 2003). In Atlantic halibut, the brain aromatase gene exhibits high expression levels during early development (Matsuoka et al., 2006).

The survival rate of the larvae after seven days of temperature treatment remained relatively high, ranging from 84.89% to 95.05%, with the lowest survival rate observed at a temperature of 35°C and the highest in the control group (<30°C). However, over the course of the 90-day rearing period, the survival rate of the juveniles decreased, ranging from 18.34% to 29.97% (at 35°C and in the control group). Several factors may have contributed to the decline in juvenile survival until the end of the rearing period, including the potential long-term effects of high-temperature treatment on the fish's vital organs, inappropriate incubation frequencies, and larvae stocking density. The dissolved oxygen and water temperature parameters remained within the normal range during the rearing period. Survival rate of 80-90% was achieved in the previous research after temperature treatment from hatching to the pre-swimming larval stage (Jun, 2021). The survival of hatched embryos decreased with increasing temperature was observed in another research, with rates of 49.5% at 27°C and 29.8% at 36°C (Rougeot et al., 2008). The survival rate of larvae ten days after treatment averaged 46.6% at 36°C and 62.1%, 81.1%, and 79.4% at 27°C, 34°C, and 35°C, respectively. By the age of 90 days, the survival rate of the juveniles ranged from 50.1% to 100% of the population of 10-day-old larvae.

Conclusion

The study results indicate that temperature differences have a significant effect ($p<0.05$) on the sex ratio of red nile tilapia (*Oreochromis niloticus*) strain. The highest proportion of male fish was obtained at a temperature of 33°C, which was 73.33%, and at 35°C, it was 64.43%. There was no significant difference ($p>0.05$) in seed survival at 7 and 90 days of cultivation.

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Conflicts of Interest
The authors declare no conflict of interest.

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