Original Article

DOI: 10.19027/jai.16.1.116-123

Aromatase gene expression and masculinization of Nile tilapia immersed in water 36 °C containing 17α-methyltestosterone

Ekspresi gen aromatase dan maskulinisasi ikan nila yang direndam hormon 17α-metiltestosteron pada suhu 36 °C

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ABSTRACT

Immersion of undifferentiated larval tilapia in high temperature and 17α-methyltestosterone (MT) can increase the male ratio. However, the effectiveness of immersion in high temperature of water containing MT remains to be evaluated. The purposes of this study were: 1) evaluate the male ratio, growth, and survival of tilapia, and 2) analyze the aromatase brain-type gene expression level in tilapia after immersing in high temperature (36 °C) containing MT at 2 mg/L for four hour with single and double immersion. Aromatase gene expression was analyzed by semi-quantitative RT-PCR (sqRT-PCR) method. The result showed that higher monosex male ratio was obtained by single immersion of MT at 36 °C at room temperature. Gene expression level of aromatase brain-type was lower on single immersion and increased significantly at second immersion compared to control (immersion at room temperature without MT). Immersion using MT and high temperature had no significant effect on fish survival. However the specific growth rate and fish biomass were higher than control. Thus, monosex male tilapia can be produced by single immersion of undifferentiated larvae at 36 °C temperature containing MT.

Keywords: male ratio, aromatase, *Oreochromis niloticus*, temperature, 17α-methyltestosterone

ABSTRAK

Perendaman larva ikan nila yang belum terdeferensiasi kelaminnya dengan suhu tinggi dan hormon 17α-metiltestosteron (MT) dapat meningkatkan nisbah kelamin jantan. Tetapi, efektivitas perendaman menggunakan MT pada suhu tinggi belum diteliti. Tujuan dari penelitian ini adalah 1) mengevaluasi nisbah kelamin jantan, pertumbuhan, dan kelangsungan hidup ikan nila, dan 2) menganalisis ekspresi gen aromatase tipe-otak pada ikan direndam menggunakan MT dengan dosis 2 mg/L selama empat jam sebanyak satu dan dua kali perendaman pada suhu 36 °C. Ekspresi gen aromatase dianalisis menggunakan metode RT-PCR semi-kuantitatif (sqRT-PCR). Hasil penelitian menunjukkan bahwa kombinasi perendaman MT satu kali pada suhu 36 °C lebih tinggi menghasilkan ikan nila jantan monoseks dibandingkan perendaman MT satu kali pada suhu ruang. Tingkat ekspresi gen aromatase tipe otak pada perendaman satu kali lebih rendah, dan meningkat secara signifikan pada perendaman kedua dibandingkan dengan kontrol (perendaman pada suhu ruang tanpa MT). Perendaman larva menggunakan MT dan suhu 36 °C tidak berpengaruh nyata terhadap kelangsungan hidup, tetapi laju pertumbuhan spesifik dan biomassa ikan perlakuan tersebut lebih tinggi daripada kontrol. Dengan demikian, ikan nila jantan monoseks dapat diproduksi dengan perendaman satu kali pada larva yang belum terdeferensiasi jenis kelaminnya menggunakan MT pada suhu 36 °C.

Kata kunci: rasio jantan, aromatase, *Oreochromis niloticus*, suhu, 17α-metiltestosteron

INTRODUCTION

Male Nile tilapia grows twice faster than the female and reaches gonadal maturity in relatively short time (Popma & Masser, 1999). Male tilapia culture could potentially increase the harvest production rather than mixed-sex. The most commonly used procedure to produce male monosex is hormonal sex-reversal that applied before fish sex differentiation into definitive male or female. The success of sex reversal treatments depends on the manipulation methods used to enhances sex steroid (androgen or estrogen). Administration of exogenous androgen can be used in producing male monosex through sex-reversal. Among androgenic steroids, 17α-methyltestosterone (MT) have been widely tested on Nile tilapia. Oral-administrated MT through feed with dose 20 mg/kg feed at 30 °C rearing temperature could produce 86.31% male (Ayuningtyas *et al*., 2015). Immersion of tilapia larva in 100 µg/L MT 10 and 13 days post-fertilization (DPF) for three hours gave 73% and 83% of male, while immersion at 200 µg/L for 14 DPF larva produced 73% male. In the same report, 91.6% male were obtained with 1,800 µg/L dose of immersion for four hours (Wasserman & Afonso, 2003).

Aside from hormonal administration, Tessema *et al*. (2006) only manipulated the rearing temperature at 36 °C for ten days and produced 86% male from 10 days post hatching larva with 100 larvae for each treatment. Sex-reversal procedure by manipulating the rearing temperature can lead to fish mortality if the temperature reach the lethal point. Higher rearing temperature can resulted in lower survival (El-Fotoh *et al*., 2014). The optimum temperature for red Nile tilapia culture is 25–31 °C (Mirea *et al*., 2013). El-Fotoh *et al*. (2014) reported that at 28 °C, male ratio reached 52.33% and 81% at 36 °C. Based on those result, temperature and sex-reversal rate are having positive correlation. Combination between MT immersion and high rearing temperature is expected to produce higher male population.

Sex differentiation in fish is controlled by gene that regulated aromatase enzyme, a cytochrome P-450 enzyme that catalysts the androgen transformation into estrogen. Aromatase correlated with sex-differentiation, reproduction and behavior and its activities are limited into certain organs in fish that related to estradiol synthesis (Callard *et al*., 2001). Aromatase gene expression also related to gonadal structure. Down-regulated expression of aromatase gene lead into testis formation and up-regulation lead to ovary formation (Sever *et al*., 1999). Analysis of aromatase gene expression can explain sex differentiation and sex-reversal mechanism in fish. This research was aimed to 1) analyze brain-type aromatase gene expression in Nile tilapia immersed with MT at 36 °C, and 2) to evaluate Nile tilapia sex-ratio, specific growth rate, survival rate, and biomass after immersed once and twice with 2 mg/L MT for four hours at 36 °C.

MATERIALS AND METHODS

Masculinization

Completely randomized factorial design was conducted in this experiment. Sex-reversal was conducted to Nile tilapia larvae by combination between hormonal exposure and temperature manipulation. In the experiment, 10 DPF Nile tilapia larvae were exposed to 2 mg/L 17α-methyltestosterone (MT; Argent, Philippines) by single immersion at room temperature (24–26 °C) and 36 °C; and double immersion at 10 and 13 DPF. Each immersion was performed for four hours with 250 larvae/treatment.

After immersion, fish were reared for two months; in the first month, fish were reared in aquariums and for the second month fish were reared in 2×1×1 m^3 hapa hanged on the soil-based pond. Feed with 40% protein were given three times a day at-satiation. Rearing water in aquariums changed every two days. Fish were reared for 60 days.

Aromatase gene expression analysis

Gene expression was analyzed before immersion, after first and second immersion, and after 60 days of rearing using sqRT-PCR method. Ten immersed larvae and five 60 days old fish were taken from each treatment for RNA extraction. Each treatment were replicated twice. Total RNA were extracted from pre-anal part (head to stomach) for larvae and gonad for 60-do fish using miRNeasy Mini Kit (QIAGEN) according to manual procedure then diluted with 30 µL DEPC 0.1%. Total RNA concentration measured using RNA/DNA calculator GeneQuant at 260 and 280 nm.

Synthesis of cDNA were performed using High Capacity cDNA Reverse Trancriptase Kit (Applied Biosystems). Total RNA was diluted

until reach 3 µg/30 µL concentration then homogenized using vortex at low-speed. RNA then incubated at 65 °C for ten min then cooled on ice for two min. RNA solution then inserted into first strand reaction mix beads tube mixed with 3 µL dT3 raceVect oligo primer (1 µg/3 µL) 5'-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT TTT TTT TTT TTT TTT TTT-3' for reverse transcriptase and let the tube stand for one min. Microtubes with the mixture were then incubated for one hour, 50 µL SDW then was added into the tubes.

Primers for brain-type aromatase gene were designed as tiArm2-F (5'-TAGGCACAGCCA GCAACTAC-3') and tiArm2-R (5'-TGGAGGAG ACGCAAACATCC-3') using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on brain aromatase sequence of Nile tilapia available on Gene Bank (Gene Bank accession code: XM005450809). Amplification performed using PCR method using following program: 94 °C for three min; 35 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s; and 72 °C for three min as described in Heriyati (2012). PCR result were separated using gel-electrophroresis in 1% agarose gel and visualized using GelRed™ on UV-light. The result then analyzed using ImageJ software, compared with β-actin as internal control.

Research parameters and data analysis

Fish-weighing were done every two weeks. In the end of rearing period, fish survival and biomass were measured. Water quality parameters measured were pH, temperature, dissolved oxygen (DO), and ammonia from aquariums and hapa. Titration method was used to measure ammonia levels, while pH, DO, and temperature were measured using electronic devices (pH meter, DO meter, and temperature meter). Water quality data were analyzed descriptively.

Sex ratio, specific growth rate, survival and fish biomass were analyzed using analysis of variance run by the SPSS v.22, and the means were compared using the Duncan test (P=0.05). Expression level of aromatase gene was analyzed semi-quantitatively using ImageJ software.

RESULTS AND DISCUSSION

Results

Aromatase gene expression

The result of sqRT-PCR of aromatase gene expression presented in Figure 1, and the result from semi-quantitative analysis is presented in Figure 2. The result showed that β-actin expression were detected in all sample and DNA bands density are relatively the same as observed from the electrophoresis result (Figure 1, bottom-left) while the bands density and expression of aromatase gene were not (Figure 1 top-right).

Expression levels of brain type aromatase gene were found lower in single immersion at 36 °C than double immersion and became lower at 60 days old fish (Figure 2). The reduction of brain type aromatase expression level indicated that enzymatic activity of aromatase was also reduced and suppressed the transformation of androgen into estrogen. Aromatase expression on 60 days old fish immersed at 36 °C was lower than control.

Male ratio

Nile tilapia male ratio was measured after 60 days to determine the success of masculinization treatment (Figure 3). Hormonal treatment with 2 mg/L MT resulted in higher male ratio than both control ($P<0.05$). Male ratio on single immersion at 36 °C was higher than room temperature ranged from 70–80%. Single and double immersion at 36 °C was not significantly different, but single immersion at 36 °C gave higher male ration than at room temperature.

Specific growth rate

Specific growth rate (SGR) on double immersion treatment was higher than single immersion (Figure 4). At room temperature, value of SGR of double immersion group was 67±0.01%/day–6.86±0.04%/day meanwhile in control group was 6.59±0.00%/day. At temperature of 36 °C, SGR was 6.85±0.00%/day–6.91±0.01%/day in double immersion treatment, whereas 6.54±0.00%/day in control treatment.

Survival

Fish survival on immersion treatment at 36 °C was not significantly different with its control (Figure 5) Control and immersion treatment at room temperature resulted in lower survival than the treatment at 36 °C. Immersion at 36 °C showed positive result for Nile tilapia survival.

Fish biomass

Fish biomass on 36 °C single and double immersion treatments were the same with single immersion at room temperature as displayed in

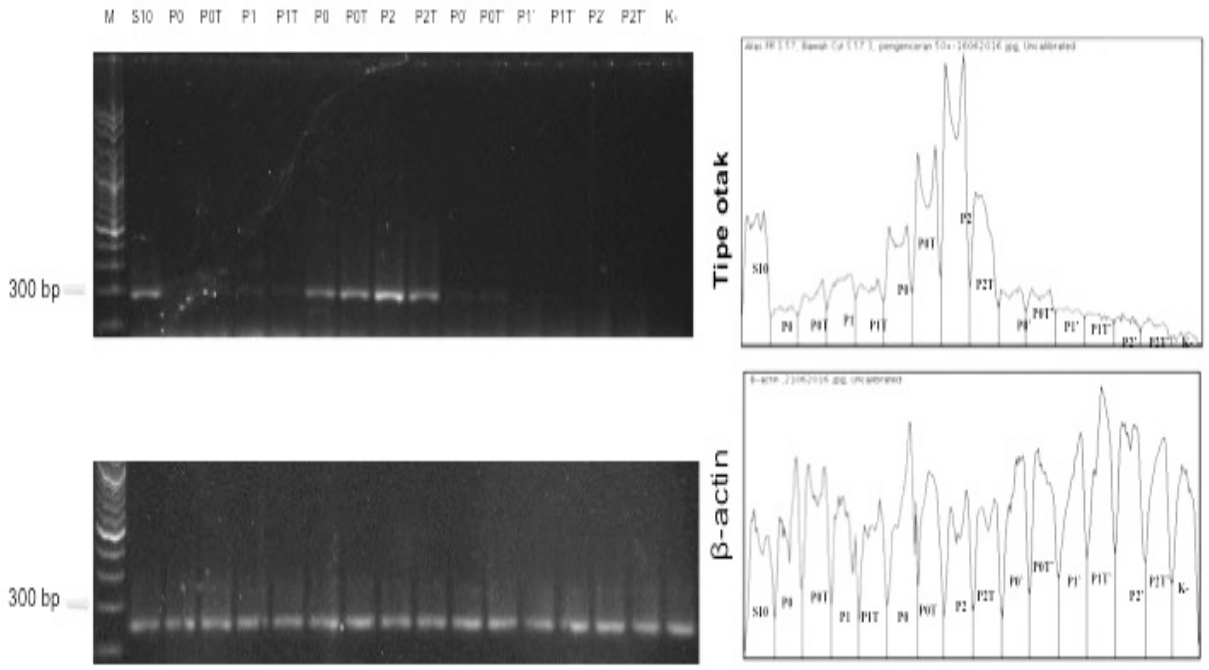

Figure1. Brain type aromatase gene and β-actin expression. M=DNA marker; S10: pre-treatment, P0: room-temperature control, P0T: 36°C control, P0T': 36°C control 2-mo, P1: single immersion at room temperature, P1': single immersion at room temperature 2-mo, P1T: single immersion at 36 °C, P1T': single immersion at 36 °C 2-mo, P2: two-times immersion at room temperature, P2': single immersion at 36 °C 2-mo, P2T: two-times immersion at 36 °C, P2T': two-times immersion at 36 °C 2-mo, K-: negative control.

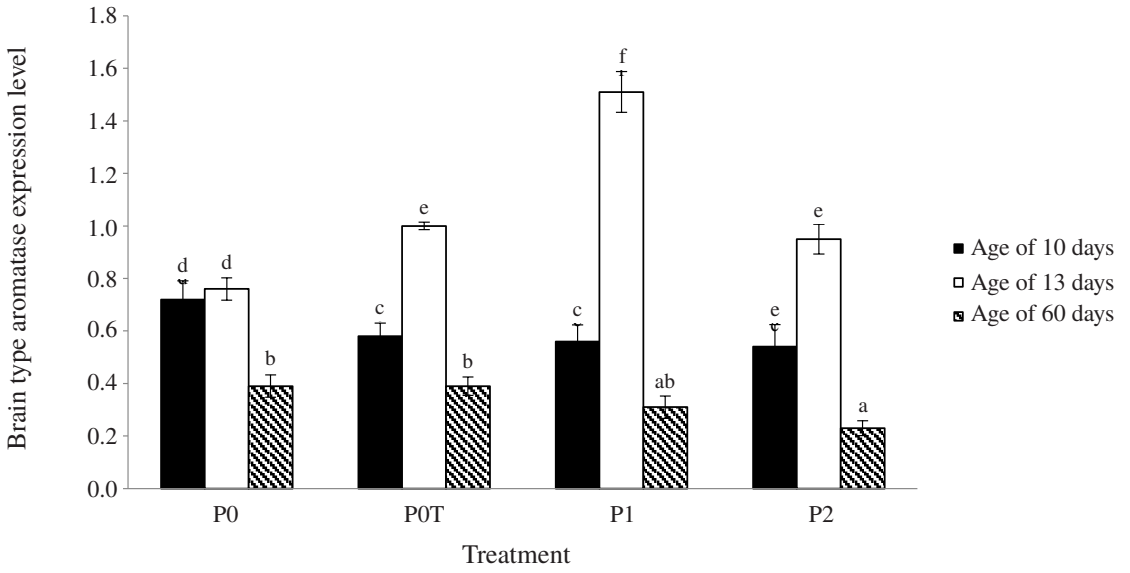

Figure 2. Brain type aromatase expression level analysis using semi-quantitative PCR. P0: room temperature control, P0T= 36° C control, P1=MT-immersion at room temperature, P2= MT immersion at 36 °C. Different lower-case letter above the SD bar showed significant difference (P<0.05).

Figure 6. Those treatment were also had higher biomass than controls.

Water quality

Temperature and pH of water were ranged optimally according to Popma and Masser (1999); 24–26 °C and 6.5–7.5 respectively. dissolved oxygen ranged 7.0–7.5 mg/L and total ammonia ranged from 0.10–0.29 mg/L.

Discussion

Aromatase expression was suppressed on single immersion treatment. But after second immersion, the expression increased then

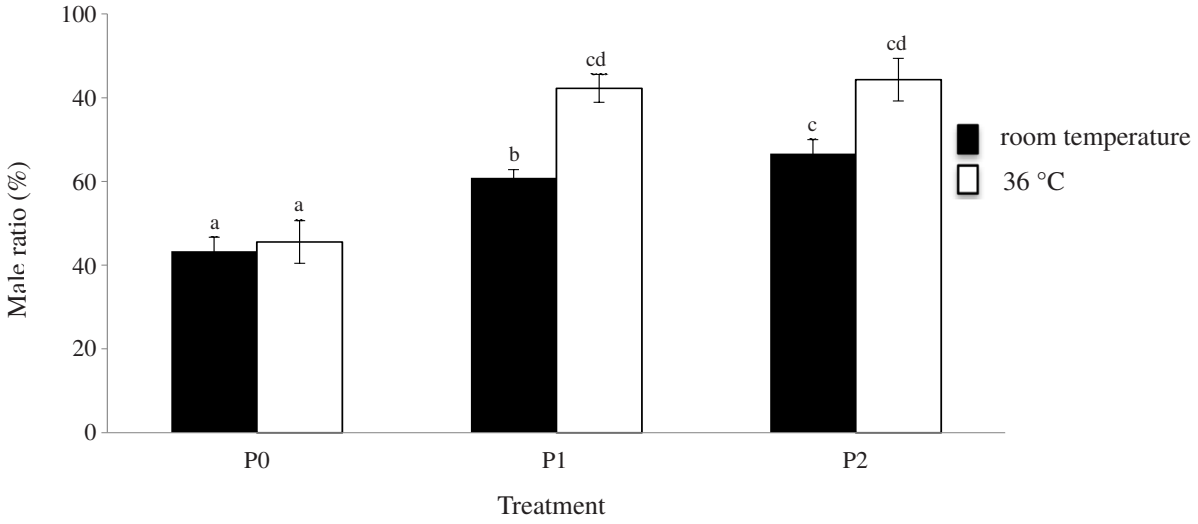

Figure 3. Male ratio from immersion treatment at 36 °C. P0= control, P1: single immersion, and P2= double immersion. Different lower-case letter above the SD bar showed significant difference (P<0.05).

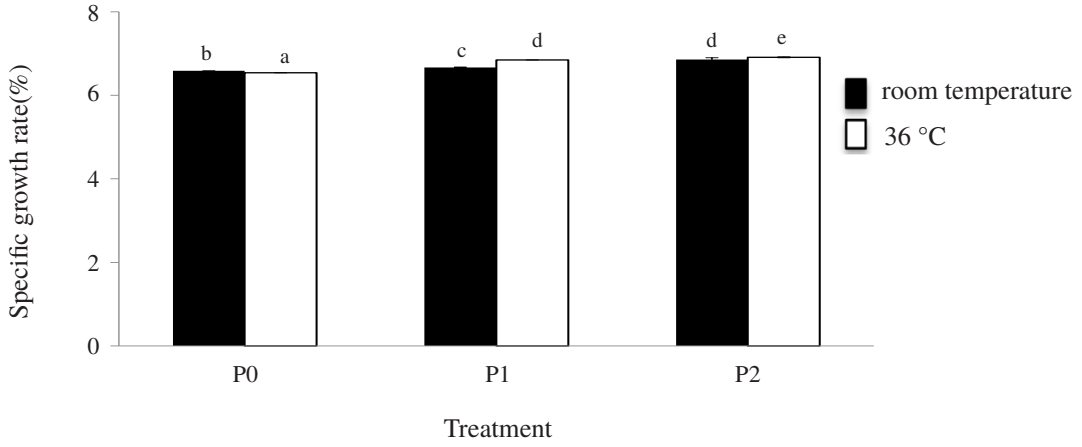

Figure 4. Specific growth rate of Nile tilapia after immersion at 36 °C. P0= control, P1: single immersion, and P2= double immersion. Different lower-case letter above the SD bar showed significant difference (P<0.05).

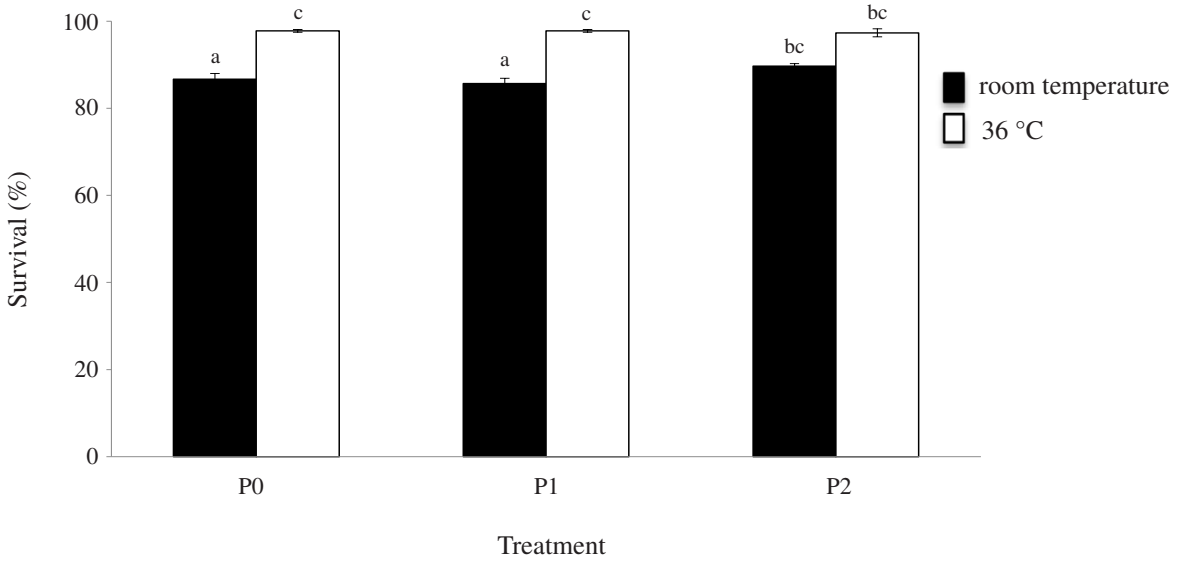

Figure 5. Survival of Nile tilapia after immersion at 36 °C. P0= control, P1: single immersion, and P2= double immersion. Different lower-case letter above the SD bar showed significant difference (P<0.05).

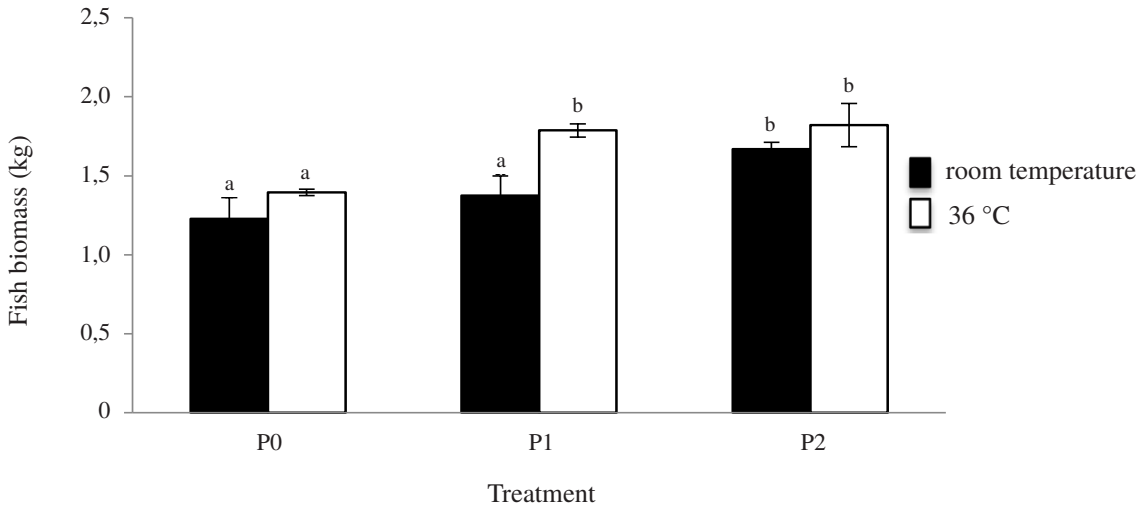

Figure 6. Nile tilapia biomass after immersion at 36 °C. P0= control, P1: single immersion, and P2= double immersion. Different lower-case letter above the SD bar showed significant difference (P<0.05).

decreased after 60 days of rearing. It possibly related with fish male-differentiation. Single immersion of MT 2 mg/L at 36 °C was more effective in suppressing aromatase gene expression than double immersion.

Azaza *et al*. (2008) reported that Nile tilapia larva that immersed at 36.9 °C resulted in higher male population (64.2–80%) and lower survival (60–81%) while larva that reared at 22, 26, 30 and 34 °C were not significantly different than control. This research also resulted in higher sex-ratio but also with relatively high survival. This research used shorter immersion duration only four hours than Azaza *et al*. (2008). Aromatase activity can be reduced by temperature (D'cottae *et al*., 2001). Sterile fish can be produced permanently using 37 °C rearing temperature for 45–60 days (Pandit *et al*., 2015). Stress from the heat affected the (gonadal) germinal cell survival at embryogenesis process.

According to Arfah and Carman (2008), the success of sex-reversal method is very dependent on environment factors that related to steroid production. The optimum time to reverse the fish sex was on sex-differentiation period. Sex differentiation is the state when the undifferentiated gonad transforms into testis or ovary based on its genetic and the process are affected by environment (Farell, 2011). Sex differentiation occurs on critical period when the embryo brain still on the bipotential state to direct the formation of gonad including its morphology, function and behavior (Carman *et al*., 2008).

Cyp19a (aromatase) gene hold an important key in sex responses to temperature. Navarro-Martin *et al*. (2011) reported that heat treatment can suppressed cyp19a (aromatase) gene expression. In fish masculinization, high temperature could inhibit cyp19a gene expression and also the enzymatic activity of aromatase. Aside from that, germinal cells of Nile tilapia were significantly reduced when the fish were reared in 32–35 °C high-temperature water (Alvarenga & França, 2009). In this research, larva immersion was performed only for four hours. Total gametes and its functionality after sex-reversal treatment were not analyzed yet. But then, in this research fish survival was higher than control in 36 °C immersion treatment.

Combination of MT-immersion (one and two times) at 36 °C resulted in the highest male percentage (Figure 3). This result was also reported by Wassermann and Afonso (2003), but with fewer fish; 125 fish/L. It was indicated that MT application was more efficient. Further research should be done to determine the maximum density of fish to reach highest male ratio. Masculinization with same method on guppy *Poecilia reticulata* Peters, with the addition of MT 60 uL/kg in feed resulted in higher male ratio than control; 55.17% to 24.30% (Soelistiyowati *et al*., 2007).

Male tilapia grows twice as fast as females (Popma & Masser, 1999). Growth rate of treated fish in this research were higher than control but less than two times. Different rearing method estimated to be the cause. Biomass of treated fish was also higher than control (Figure 6). Survival (Figure 3) and growth (Figure 4) were higher than control, resulted in higher biomass.

Specific growth rate was significantly different between hormonal immersed and control. Single and double immersion gave higher growth than control. This indicated that immersion with MT could increase fish growth. Fish growth are generally affected by feed quality, zooplankton abundance, water quality and stocking density (Soelistiowati *et al*., 2010). MT immersion increased proteolytic activity in common carp resulted in high growth rate. MT immersion also triggering thyroid, internal function, and insulin secretion from fish pancreatic B cell (Ajiboye *et al*., 2015). Androgenic steroid can help the releasing process of growth hormone from fish hypophysis. Anabolic steroid can be potentially becomes beneficial compound in aquaculture by increasing fish weight and muscle deposit (Ajiboye *et al*., 2015).

Environment temperature can affected fish biomass by changing its metabolism (Liana 2007). If the body metabolism was increased, feed intake also increased. Reported by Pandit (2010), when the rearing temperature raised to 32 °C, fish feed consumption also increased. Single and double MT immersion at 36 °C not affected Nile tilapia survival. This result indicated that immersion with this treatment were not negatively affected larva and post-larva survival until 60 days old. Yustina *et al*. (2012) stated that sudden changes on rearing temperature can lead to stress and mortality.

CONCLUSION

Single immersion in 2 mg/L 17α-methyl -testosteron at 36 °C was effective to suppress brain type aromatase gene expression and also gave high male ratio, specific growth rate, fish biomass, and survival.

ACKNOWLEDGMENT

This study was partially funded by grants from Bogor Agricultural University, Indonesia (PSU: 696/IT3.11/PN/2016).

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