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Masculinization of nile tilapia (*Oreochromis niloticus*) using extract of bull testes

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Abstract. Tilapia males grow faster to increase the production of the fish, there is a necessity to produce all males by using extract of bull testes. The research method used in this study was the experimental Completely Randomized Design (CRD) with five treatments and three replications. The treatments were A: without immersion and without oral treatment (control); B; immersion 600 $\mu g \cdot L^{-1}$ without oral treatment; C: immersion 600 $\mu g \cdot L^{-1}$ and oral at a dose of 30 $mg \cdot kg^{-1}$ feeds, D: immersion 600 $\mu g \cdot L^{-1}$ and oral at a dose of 40 $mg \cdot kg^{-1}$ feeds, E: immersion 600 $\mu g \cdot L^{-1}$ and oral at a dose of 50 $mg \cdot kg^{-1}$ feeds. Results showed that the use of bull testicle extract mixed to media at concentration of 3 ml L^{-1} produced red male nile of 69.07 %. In addition, the immersion of the extract at 500 $\mu g \cdot L^{-1}$ resulted the male of 86.71 %. Sex reversal by dipping at 600 $\mu g \cdot L^{-1}$ 17α-*methyltestosterone* combined with oral administration at 40 $mg \cdot kg^{-1}$ produced the highest male of Nile Tilapia Java carp (86.67 %) compared with dipping, which produced 65.56 % of male. These results suggested that combination of dipping and oral is more effective for sex reversal.

1. Introduction

Nile Tilapia is one of freshwater commodities that has a high economic value, easy to culture and has a thick body flesh so it is suitable to be used as refined products, one of them is fillet. Tilapia fillet has very wide export market, especially in the United State [1].

Innovation and technology are needed to increase production and productivity of tilapia culture to fulfill market demand. One of technology innovations which can be conducted is selective breeding to obtain good strain, one of the good tilapia strains is Nirwana (Nila Ras Wanayasa).

Nile Nirwana is the strain resulted from the Development Center for Tilapia and Carp Wanayasa, Purakarta. The advantage of nirwana is faster growth than other strains. Culture of larvae until it reaches a weight more than 650 g can be done in 6 month, in addition nirwana has also thick flesh compare with other strain [2]. However, its production is still can be increased by masculinization using natural hormone obtained from bull testicle.

Growth rate of male tilapia is faster than female one, so it is important to develop a technique to produce male monosex of tilapia by administration of male hormone (testosterone). The technique usually use synthetic hormone 17α-*methyltestosteron* to revers fish sex [3, 4]. However the distribution of synthetic hormone is limited, so the price is high. Hormone residue might become an environment pollutant [5]. One alternative to produce male tilapia with masculinization is by using environmental friendly natural testosterone originated from bull testicles. The purpose of this research was to increase the production of Nile tilapia by masculinization with oral and dipping administration of the bull testicle extract.

2. Materials and Methods

2.1. Materials

The materials used in this research:

a) Nirwana tilapia originated from The Development Center for Tilapia and Carp Wanayasa, Purakarta, West Java. Male and female broodstocks weight was in the range of 300–400 g and 200–300 g, respectively.
b) The testes of bull was obtained from the slaughter house in Ciroyom Bandung.
c) Ether solution for immersion the bull testicles.
d) ETTS was made from the fresh testicle of bull or local male cow. The testicle tissue was chopped into a size of 1 mm^2. The chopped testicle was dried by freeze dryer at a temperature of -80 $^{\circ}C$ to obtain powder.
e) Acetocarmine solution is obtained by dissolving 0.6 g carmine into 100 mL acetic acid 45 % for staining fish gonadal tissue in sexing.
f) 'Artha' feed contained 25 % protein.

2.2. Methods

The research method used in this study was the experimental Completely Randomized Design (CRD) with five treatments and three replications. The treatments were:

A: without immersion and without oral treatment (control)
B: immersion at 600 $\mu g \cdot L^{-1}$ without oral treatment
C: immersion at 600 $\mu g \cdot L^{-1}$ and oral administration at 30 $mg \cdot kg^{-1}$ feed
D: immersion at 600 $\mu g \cdot L^{-1}$ and oral administration at 40 $mg \cdot kg^{-1}$ feed
E: immersion at 600 $\mu g \cdot L^{-1}$ and oral administration at 50 $mg \cdot kg^{-1}$ feed

$$Xij = \mu i + \tau j + \varepsilon ij$$

Xij = result of observation on treatment to -i replication to-j
μi = general average
τj = the effect of treatment to-i
εij = effect of random factors treatment to-i replication to-j

2.3. Procedure

Fifteen aquaria contain 20 L water were prepared, each aquarium was equipped with aeration for oxygen supply from the blower. Another a set of fifteen 60 L-aquaria were prepared to raise the masculinized larvae. When the fish reached 3 cm of total length, they were transferred to the net cage in a pond.

One day old tilapia larvae or at eye spot phase were treated with an extract of bull testicle powder (ETTS). The extract of hormone was poured into the water where the larvae were reared. The larvae were immersion in the extract of hormone for seven days.

Until the yolksack larvae were depleted, the larvae were fed with yolksack of a chicken egg. Then the larvae were fed with natural food, namely Chlorella and Artemia. Two weeks old larvae were then fed with proper commercial feed. After one month old, the treated fish were then placed in floating cages and fed with commercial feed with the amount of five percent/body weight/day, three times a day. Fish sampling to measure growth and to adjust the amount of feed to be given, is done every ten days in three months.

In the third month, survival and growth rates were calculated. In addition, thirty fish from each treatment were sexing. Sexing was conducted by analyzing the gonad stained with *acetocarmine*. The results were grouped into three, namely male, female and intersex.

2.4. Parameters

2.4.1. Survival rate. Survival Rate was observed every day by counting and removing the dead fish. The calculation of survival rate was done by using the formula [6]:

$$SR = \frac{Nt}{No} \times 100\ \%$$

SR = Survival Rate (%)
Nt = the amount of fish of three months age
No = the amount of fish of the initial rearing

2.4.2. Growth. Absolute growth using formula:

$$GA = Wt - Wo$$

GA = absolute growth
Wt = fish weight by three months old
Wo = initial weight

Specific growth rate using the formula:

$$SGR = \frac{(Lnt - LnWo)}{T} \times 100\ \%$$

SGR = Specific Growth Rate (%)
Wt = biomass of fish weight in the end of study (g)
Wo = biomass of fish weight in in the end of study (g)
T = observation periode (day)

2.4.3. Sex ratio: male, female and intersex. At the end of the research, gonad of thirty fish from each treatment were analyzed. The gonad was placed in a petri dish, chopped and mixed with acetocarmine. The gonad was then analyzed under a microscope to determine whether the fish was male, female or intersex. The formula is:

$$M = Gm\ s^{-1} \times 100\ \%$$
$$F = Gf\ s^{-1} \times 100\ \%$$
$$I = Gi\ s^{-1} \times 100\ \%$$

M: Male, F: Female, I: Intersex, s: amount of sample, Gm: male fish, Gf: female fish, GI: Intersex fish

2.5. Data analysis
Survival rate, growth rate and sex ratio were analyzed statistically using F test and Duncan test. Water quality parameters were compared with existing standards.

3. Result and Discussion

3.1. Result

3.1.1. Percentage of male. The result of this research showed that the production of male in the range from 25.55 to 86.67 %. The highest of male percentage of Nirwana was obtained from D treatment (600 $\mu g \cdot L^{-1}$ + 40 $mg \cdot kg^{-1}$ feeds), that reached 86.67 %. The lowest percentage of Nirwana male was 25.55 % obtained in a treatment (without hormone).

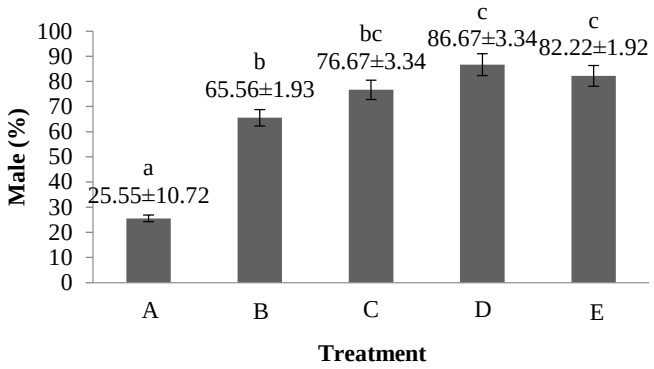

Figure 1. Average Percentage of Male in Tilapia treated with various dosages of bull testicle extracts.

D treatment produced high percentage of male and significantly different from treatment A (without hormone) and B (600 $\mu g \cdot L^{-1}$ + 0 $mg \cdot kg^{-1}$ feeds) (p, 0.05) but insignificantly different to C treatment (600 $\mu g \cdot L^{-1}$ + 30 $mg \cdot kg^{-1}$ feeds) and E (600 $\mu g \cdot L^{-1}$ + 50 $mg \cdot kg^{-1}$ feeds). In normal spawning, tilapia usually produced less than 50 % male. It means that the addition of bull testicle extract into feed and into the media increased the percentage of males.

3.1.2. Percentage of female. Percentage of female obtained from the research ranged from 12.22–74.45 %. The high percentage of the female was produced from a treatment (Without hormone) that reached 74.45 %. In contrast, the low percentage of female was produced from the treatment (600 $\mu g \cdot L^{-1}$ + 40 $mg \cdot kg^{-1}$ feed) 12.22 % (figure 2).

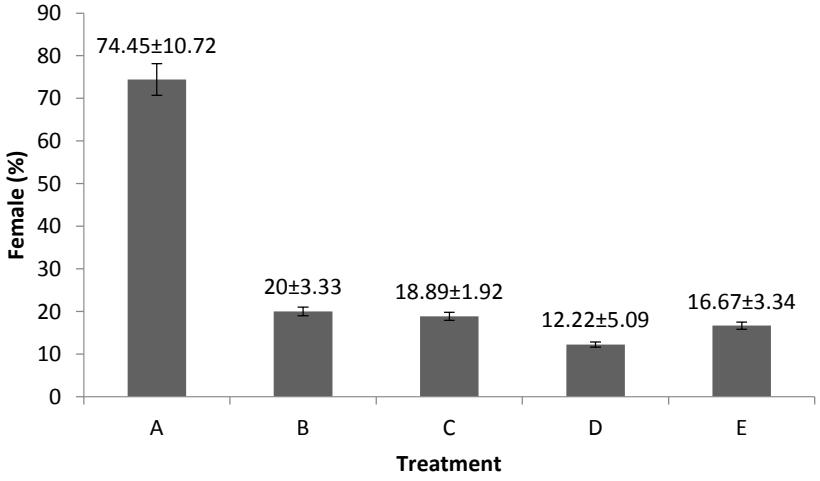

Figure 2. Average percentage of female in Tilapia treated with various dosage of bull testicle extracts.

3.1.3. Percentage of Intersex. Percentage of nirwana intersex obtain from the research ranged from 0 % to 14.44 %. The highest (14.44 %) Nirwana intersex production was obtained from B treatment (600 $\mu g \cdot L^{-1}$ + 0 $mg \cdot kg^{-1}$ feeds). Meanwhile the lowest one (0 %) was obtained from a treatment (without hormone).

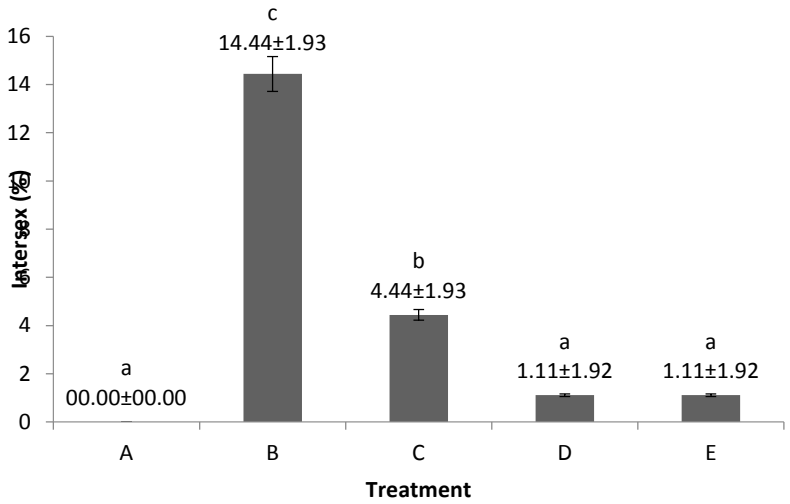

Figure 3. Average Percentage of Intersex in Tilapia treated with various dosage of bull testicle extracts.

3.1.4. Nirwana sex ratio. The data of nirwana sex ratio is presented in table 1.

Table 1. Average percentage of sex rasio in tilapia treated with various dosage of bull testicle extracts.

Treatment	Percentage (%) male	Percents (%) Female	Percents (%) Intersex
A: 0 µg·L^{-1} + 0 mg.kg^{-1} of feed	25.55	74.45	0
B: 600 µg·L^{-1} + 0 mg·kg^{-1} of feed	65.57	20	14.44
C: 600 µg·L^{-1} + 30 mg·kg^{-1} of feed	76.67	18.89	4.44
D: 600 µg·L^{-1} + 40 mg·kg^{-1} of feed	86.67	12.22	1.11
E: 600 µg·L^{-1} + 50 mg·kg^{-1} of feed	82.22	16.67	1.11

Treatment D at a dose of 600 µg·L^{-1} + 40 mg·kg^{-1} of feed produced the highest male fish hatching percentage of 86.67 %. Treatment A without incorporating the hormone (control) produced the highest female fish percentage of 74.45 %. Treatment B at the dose of 600 µg·L^{-1} + 0 mg·kg^{-1} feed produced the highest intersex fish percentage in 14.44 % (table 1).

3.1.5. Survival rate. The statistical analysis result showed that there was no significant difference among treatments (figure. 4). The highest survival percentage of 69.50 % were found in treatment D (600 µg·L^{-1} + 0 mg·kg^{-1} feeds), while the lowest survival percentage of 46.33 % was found in treatment C (600 µg·L^{-1} + 30 mg·kg^{-1} feeds) (figure. 4).

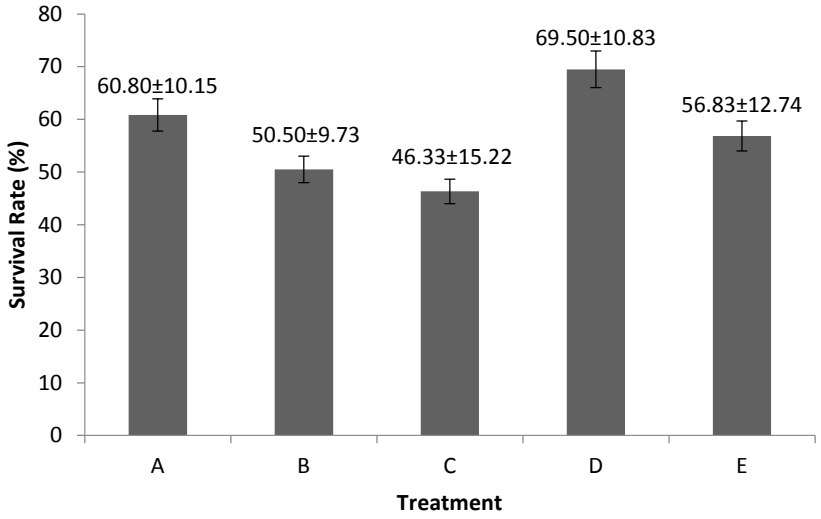

Figure 4. Survival rate of Tilapia treated with various dosage of bull testicle extracts.

3.1.6. Average length and weight of Tilapia fish. Weight and length of tilapia were measured when they got three months old or when the gonads were ready to be observed. This study indicated that treatment a produced fish with average maximum length of 6.18 cm average maximum weight of 3.09. Treatment D produced fish with average minimum length of 4.98 cm and average minimum weight of 1.75 (figure 5).

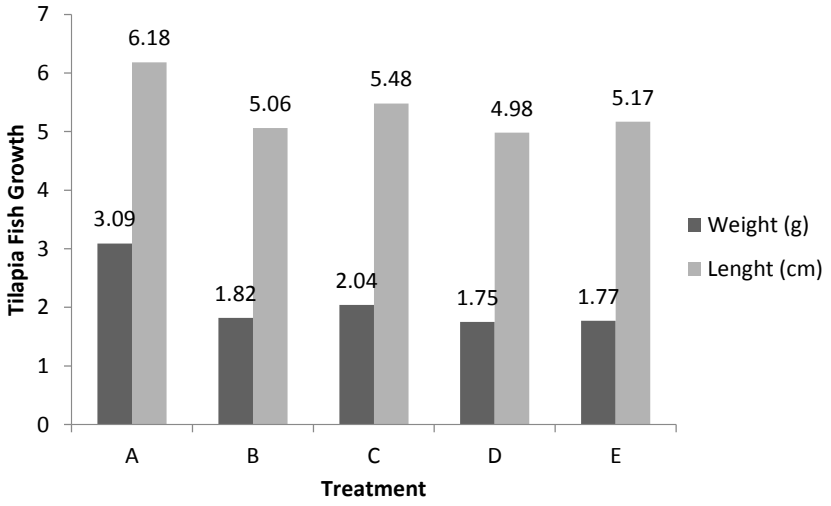

Figure 5. Growth of Tilapia obtained from masculinization with bull testicle extract.

The statistical analysis showed that treatment A (without hormone administration) produced fish with the highest average length and weight, being different significantly from treatment, B, C, D and E (table 2). The statistical analysis showed that treatment A (without hormone administration) produced fish with the highest average length and weight, being different significantly from treatment, B, C, D and E (table 2).

Table 2. Average length and weight of tilapia fish.

Treatment	Average Weight (g)	Average Length (cm)
A: 0 $\mu g \cdot L^{-1}$ + 0 $mg \cdot kg^{-1}$ feeds	3.09 ± 0.27^{b}	6.18 ± 0.17^{d}
B: 600 $\mu g \cdot L^{-1}$ + 0 $mg \cdot kg^{-1}$ feeds	1.82 ± 0.08^{a}	5.06 ± 0.08^{ab}
C: 600 $\mu g \cdot L^{-1}$ + 30 $mg \cdot kg^{-1}$ feeds	2.04 ± 0.24^{a}	5.48 ± 0.21^{c}
D: 600 $\mu g \cdot L^{-1}$ + 40 $mg \cdot kg^{-1}$ feeds	1.75 ± 0.09^{a}	4.98 ± 0.10^{a}
E: 600 $\mu g \cdot L^{-1}$ + 50 $mg \cdot kg^{-1}$ feeds	1.77 ± 0.10^{a}	5.17 ± 0.06^{b}

3.1.7. Water quality. Table 3 shows that all parameters of water quality in this research were still in the optimum range for nile tilapia culture according to Indonesia National Standards/Standar Nasional Indonesia (SNI). The data of water quality is presented in table 3.

Table 3. Average water quality during tilapia rearing.

Treatment	Average Temperature (^{o}C)	Average of pH	Average Dissolved Oxygen ($mg \cdot L^{-1}$)	Average Ammonia ($mg \cdot L^{-1}$)
A: 0 $\mu g \cdot L^{-1}$ + 0 $mg \cdot kg^{-1}$ feeds	25.7	7.2	6.7	0
B: 600 $\mu g \cdot L^{-1}$ + 0 $mg \cdot kg^{-1}$ feeds	24.5	7.1	6.1	0.001
C: 600 $\mu g \cdot L^{-1}$ + 30 $mg \cdot kg^{-1}$ feeds	23.5	7.3	6	0.001
D: 600 $\mu g \cdot L^{-1}$ + 40 $mg \cdot kg^{-1}$ feeds	23.0	7.5	5.8	0.003
E: 600 $\mu g \cdot L^{-1}$ + 50 $mg \cdot kg^{-1}$ feeds	25	7.6	5.5	0.003
Indonesia National Standards	25 to 32 $^{\circ}C$	6.5 to 8.5	> 3	< 0.002

3.2. Discussion

The treatments of D and E resulted female percentages of 86.67 % and 82.22 %, respectively. This result suggested that the concentration of testosterone entering the larva's body through osmotic and diffusion processes was sufficient to reverse the larva sex to be male. It can be sound that percentage of Nile male was higher when the concentration of bull testicle extract powder (EBT) increased.

The A treatment or control produced the lowest male percentage (25.55 %) because there was no addition testosterone entering to the fish's body, so in the process of sex reversal there was no supporting factor to reverse female to be male. Although concentration bull testicle extract powder (EBT) was increased, such as in treatment E (600 $\mu g \cdot L^{-1}$ + 50 $mg \cdot kg^{-1}$ feed) resulted the male percentage of 82.22 % lower 4.45 % comparing with D (600 $\mu g \cdot L^{-1}$ + 40 $mg \cdot kg^{-1}$ feed) treatment. The decrease in male percentage might be caused by an excessive concentration of bull testicle extract powder (EBT). A Treatment at dose of 600 $\mu g \cdot L^{-1}$ + 40 $mg \cdot kg^{-1}$ feed was assumed as a maximum dose which resulted high male percentage.

The high production of nirwana male from this research occurred because of testosterone containing in the EBT. This hormone is effective to maintain spermatogenesis process and a main mediator in spermiation [7]. Exogenous testosterone hormone entering the fish's body will affect hypothalamus to release LH and FSH. While LH stimulates by Leydig cell to produce testosterone. Fish affect Sertoli cell to stimulate CAMP (Cyclic Adenosine Mono Phospat). Which induces the ABP (Androgen Binding Protein). The ABP has a function to bind testosterone release by Leydig cells. The bind the complex will interact directly in second meiosis, which change secondary spermatocyte to be spermatid.

Extract of bull testes (EBT) contains steroid, ie androgen. This hormone is responsible for male gonadogenesis, testicle. The more EBT added into feed, the more males produce and the concentration of 40 $mg \cdot kg^{-1}$ feed yields the highest males [9]. Beyond 40 $mg \cdot kg^{-1}$ feed, it occurs the phenomenon of paradoxical effect. Previous work using only immersion of 600 $\mu g \cdot L^{-1}$ produces less than 75 % male.

Statistical analysis indicated that a treatment (without hormone) produced a high percentage of females, significantly different from other treatments. There is no paradoxical affect a course when the use of hormone is too high, then the reproduction of male will not be rich, even reproduction of female will increase. Except exogen hormone causes testosterone synthetic in aromatase inhibition stopped. Again, it shows that in normal spawning tilapia produces much females than males. And the treatment of immersion larvae in EBT combined with oral treatment, feed mixed with EBT, reduced the number of female dramatically until 12 %.

Intersex fish was found because the effect of EBT in masculinization proses was not single effect. It was proved in the B treatment (600 $\mu g \cdot L^{-1}$ + 30 $mg \cdot kg^{-1}$ feed). In this treatment the larvae was only immersed in water mix with 600 $g \cdot L^{-1}$ for twelve hours starting from eye spot phase. Without feeding with hormones-containing food. The use of EBT, also showed the phenomenon of intersex. Intersex fish has ovarium and testicle in one gonad. The dose of 30 mg EBT kg^{-1} feed is not sufficient to alter the gonad into males. The higher oral dose was able to produce less intersex fish.

In terms of time efficiency and treatment as well as the amount of hormone used, immersion is considered to be the most efficient method. However, it has not been fully effective in producing a higher percentage of male fish seeds. Steroid hormone used in the process may leach into the water [8]. In addition, excessive steroid hormone usage and prolonged immersion may cause a paradoxical effect on fish. This indicates that monosex fish, seeds produced by masculinization through immersion is not very effective and, therefore, an additional method is required in order to attain the higher per cent of male fish seeds. The process of masculinization through immersion can be incorporated with oral administration of feed [9].

The survival rate of each treatment did not vary significantly, which suggested that bull testicle extract in each treatment did not considerably affect the survival rate of the fish. The dosage of hormone was given within the safe dose limit, so that it did not affect the survival of the fish. It means that the addition of EBT into the media and in the feed did not give any harmful effect to the fish.

The water temperature fluctuated between 23.0–25.7 °C during the research, which was stills a suitable range of temperature for the growth of tilapia. The optimum water temperature for growing tilapia fish larvae is around 25–32 °C [10]. Optimum temperature in fish breeding will influence the digestive enzymes and metabolism. High level consumption of feed combined with effective digestion and metabolism process will produce optimum energy for growth and reproduction [11].

pH value (Power of Hydrogen) is the hydrogen ion value in water. Water with high H+ ion content will be acidic and water with low H+ ion content will be alkaline. The determining factor in water quality is the acidity (pH). The pH value during the research ranged between 7.2–7.6, which was good for the growth of tilapia fish. Optimum pH range for growing tilapia fish is between 6.5–8.5 [12]. The low temperature-liquid will decrease the activity of the body or cause fish to become weak, easily get infected and usually followed by the high rate of mortality [13].

Dissolved oxygen content during the research fluctuated between 5.5–6.7 $mg \cdot L^{-1}$. Based on DO needed to sustain tilapia fish seeds is > 3 $mg \cdot L^{-1}$ [10]. Oxygen availability is one of determining factors that contribute to fish fed consumption (appetite) since oxygen is required to turn food into energy. A decrease in appetite leads to low consumption of feed, which cause fish to lack the energy needed for growth. Low dissolved oxygen causes a slowing down in fish growth.

The level of amino concentration during the research was 0–0.003 $mg \cdot L^{-1}$. Ammonia (NH_3) is the main substance generated by protein decomposition, which is toxic for fish. Therefore, NH_3 concentration in water should not be more than 1 ppm [14]. The source of ammonia in water is the breakdown of nitrogen (protein and fertilizer) and inorganic nitrogen contained in soil and water [15].

4. Conclusion

Based on the results of research, it can be concluded: Immersion with bull testicle extract of 600 $\mu g \cdot L^{-1}$ combined with oral administration of 40 $mg \cdot kg^{-1}$ feed produces the highest level of male fish percentage (86.67 %) and the highest survival rate (69.50 %).

Suggestions

Based on the result of the research, it can be suggested that in order to attain the maximum amount of male fish population, immersion and oral administration can be incorporated, with the dosage of bull testicle extract of 600 $\mu g \cdot L^{-1}$ and 40 $mg \cdot kg^{-1}$ of feed.

Acknowledgements

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