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Research Article

Synodontis eupterus Larvae Masculinization Using Javanese Long Pepper Extract (*Piper retrofractum*)

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ABSTRACT

Synodontis eupterus male represents only 5-10 % of farmer total synodontis production, which limits male availability for reproduction purpose. A method widely used in overcoming the low male production was masculinization through synthetic hormones, which was restricted due to its adverse impact on the environment. Natural ingredients from plant were demonstrated in various studies to substitute the 17α-methyltestosteron hormone on masculinization. This research aimed at evaluating the effects of Javanese long pepper extract (JLP) through immersion on synodontis fish larvae. The research was conducted using factorial design with two faktor (4x2) consisted of eight treatments: 17α-methyltestosteron 2 mg L^{-1} (MT) immersion for 5 and 1^0 hours, JLP dose 0.125 mg L^{-1} (P1) immersion for 5 and 10 hours and dose 0.25 mg L^{-1} (P2) immersion for 5 and 10 hours compared to the control treatment without immersion (P0). A hundred of synodontis larvae of 10 days old after hatching each replication were used in the immersion treatments. The results showed that JLP treatments produced 25-40 % of male synodontis age four months, 1-2 % females and above 60 % intersex. While at age five months, the percentage of intersex fish decreased to 20-40 %, the female fish increased to 60-62 %, and male fish ranged 20-35 % in JLP compared to the control P0 (15 %). The dose of JLP 0.25 mg L^{-1} increased mortality of about 14-54 %.

Keywords: *Masculinization, larvae Synodontis eupterus, Javanese long pepper, 17α-methyltestosteron, survival rate.*

1. Introduction

Synodontis eupterus is a freshwater ornamental fish of which the origin is the Niger river, Africa (Alderton 2008). Synodontis is being widely produced in Indonesia in order to fulfill both local and export demands (Priyadi *et al.* 2010). However, its production is limited as a result of the male synodontis availability. Indeed, *S. eupterus* male represents only 5-10 % of the total population. A method used to overcome the mentioned constraint is male sex formation technique (masculinization). Hormone that is generally used for masculinization is 17α-metiltestosteron hormon (MT) (Zairin 2002). Nonetheless, the use of MT was restricted by the government because it does not easily decompose, is carcinogenic and contaminates the surrounding environment (Wiryowidagdo 2005).

Natural hormones that are derived from animals were proven to be used for masculinization such as cow testes meal extract, which resulted in producing 85.6 % male tilapia through immersion method at a dose of 5 ml L^{-1} (Iskandar, 2010). Similar results were also reported in freshwater prawn (*Macrobrachium rosenbergii*). Arisandi (2007) stated that sea cucumber steroid extract at doses of 1 mg L^{-1}, 2 mg L^{-1} and 3 mg L^{-1} in juvenile freshwater prawn could result in an increase in male freshwater prawn population compared to the negative control.

Steroid as sex hormone is not only derived from animal, but also from plant, commonly reffered to as phytosteroids (Cseke *et al.* 2006). Phytosteroid that helps in differentiation, male sex formation and secondary sex characteristics is known as phytoandrogen. Masculinization using phytoandrogen has been already proven in previous studies. For instance, *Lunasia amara* at a dose of 20 mg kg^{-1} feed in Siamese fighting fish (*Betta splendens*) resulted in producing 70.7 % male (Alfian 2003). In addition, purwoceng (*Pimpinella alpina*) extract

at a dose of 20 mg L^{-1} in black tilapia (at the age 4 days post-hatched) was also observed to increase the male ratio up to 73.3 % (Putra 2011). Supplementation of long Jawa chili extract in Siamese juvenile catfish (26±1.6 g) and potential broodstock (250±18.6 g) through feed at 37.5 mg and 187.5 mg kg $fish^{-1}$ day^{-1} during 8 months resulted in increasing gonad maturity index and testosterone level in blood (Elisdiana 2015).

Javanese long pepper (*Piper retrofractum*) is referred to as a plant that is being widely used in Indonesian traditional medicine (Usia 2012), and is produced in places such as Jawa, Madura, Bali and Maluku (Rukmana 2006). Javanese long pepper fruit includes diverse compounds such as free amino acids, resins, essential oils (tepenoid), n-octanol, linalool, terpinil acetate, sitronelil acetate, citral, saponnins, polyphenols, resins (kavisin), β-citosterol, and some types of alkaloid such as piperine, piperidine, piperatin, piperlonguminine, sylvatine, guineensine, piperlongumine, filfiline, sitosterol, methyl piperate (Usia 2012). Chemical compounds derived from steroids, saponins, alkaloid and tannin act as aphrodisiac engendering androgenic and anabolic effects (Moeloek *et al.* 2010). This research aimed at evaluating the effects of Javanese long pepper extract (JLP) through immersion on synodontis fish masculinization.

2. Methode

Time and place

The present research was conducted between June and December 2016 in teaching farm, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. JLP extraction was performed in the laboratories of plant research test and medicine center (Balittro), Bogor. Gonad identification was performed in the laboratory of reproduction and fish genetics, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University.

Research design

A completely randomized factorial design (2x4) with three replicates was used in the present study. The treatments consisted of immersion duration (5 and 10 hours), Javanese long pepper extract JLP doses i.e. 0.125 mg L^{-1} (P1) and 0.25 mg L^{-1}(P2), and controls i.e. negative control (without both MT and JLP) and positive control (2 mg L^{-1} MT).

Materials

Synodontis larvae, at the age of 10 days post hatching and at average body weight and length of 0.023 gr $fish^{-1}$ and 0.7 cm (respectively), and each replicate had 100 fish. Part of the plant that was used in the extraction process was Ripped Jawa chili fruit from Kayu Manis area, Bogor. The fruit was first dried and grinded in order to obtain chili powder, which was then placed in a maserator (for 2-3 hours) and mixed with 95 % ethanol at a ratio of 1:5 (500 ml of ethanol and 100 g of chili powder), and kept 24 hours. The maserator product was then squeezed and filtered using a filtering paper till dregs and filter were obtained. The filter was then vaporized with a rotavator in order to separate the solvent from the thick JLP.

Immersion treatment and rearing period

Synodontis larva were immersed according to the doses of each JLP treatment (P0, P1, P2) and MT, and the immersion duration (5 and 10 hours). After immersion, larvae were transferred into rearing aquariums (29 x 30 x 30cm) and fed on artemia naupli (at satiation) four times a day i.e. 04.00 a.m., 11.00 a.m., 5.00 p.m. and 10.00 p.m. After a week, larvae were then fed on worms (ad libitum) till the fifth week of the experiment, after which the fish were fed on commercial feed (34.37 % protein). Commercial feed was provided three times a day, at 6.00 a.m., 12.00 a.m., and 6.00 p.m. (*at satiation*). After the 13^{th} week, fish were transferred into larger aquarium (50 x 60 x 50 cm). During the research, siphoning was performed and water was exchanged 10 % daily and 30 % weekly. At the age of 3 months, a weekly water exchange of 70 % was applied. Water quality parameters such as temperature (25-27 °C), pH (6.5-7.4) and dissolved oxygen (4.9-5.8 mg L^{-1}). Sampling on body weight and total body length were performed every two weeks during 14 weeks, while survival rate was observed a week after immersion and on the 16^{th} week of the rearing period. Gender identification was performed once the fish was 4-5 months of age by sampling out 30 % of the fish in each treatment. Samples were identified through a coloration method using acetocarmin (Zairin 2002).

Data analysis

The data were analyzed using Microsoft Excel 2013. Data on SR and gender percentages were analyzed using SPSS 22. Significant different treatment were further analyzed was followed by a Duncan's post-hoc comparison test at a confidence level of 95 %. Data on water quality and growth were analyzed descriptively.

3. Results and discussion

Survival rate

S. eupterus larvae Survival rate (SR) a week after immersion in the 0.25 mg L^{-1} JLP treatment was lower compared to other treatments (between 49.67 % and 92.33 %) with immersion duration of 5 and 10 hours (**Table 1**). Thereby, *S. eupterus* fish Survival rate (SR) after a period of 4 months showed that the 5 and 10 hours immersion treatments at a JLP dose of 0.25 mg L^{-1} were the lowest i.e. 46.33 % and 86,33 % (*p<0.05*), no significant differences were oberved in other treatments with different immersion duration, ranging between 88.33 % and 96.67 %. Observation results of synodontis larva survival rate a week after immersion at a dose of 0.25 mg L^{-1} JLP (10 h immersion) resulted in the lowest SR being 49.67 %. Significant differences were also observed in the 0.25 mg L^{-1} JLP treatment for both 5 and 10 h immersion durations. Based on observations, fish death was caused by growing air bubbles in the fish that kept growing during the immersion process accompanied by convulsions. The 10 h JLP immersion treatment at a dose of 0.25 mg L^{-1} resulted in the lowest SR, indicating that the dose might be too high. Similar results were reported by Wahjoedi *et al.* (2004). They reported that the LD_{50} value of javanese long pepper fruit is 3.32 mg 10 g $weight^{-1}$ mice weight, and a higher dose would result in fish death. The component that can cause fish death in JLP is piperine, which can inflict toxic effects with insecticide qualities (Scott *et al.* 2008). It also functions as neurotoxin and causes knockdown and fast insects death (Scott *et al.* 2008). In addition, saponin compound of JLP is also toxic to cold-blooded animals such as fish, snails and insects (Tekeli *et al.* 2007). No significant differences were observed between the 4 months old fish and the one week old larva (after immersion) in terms of survival rate, signifying that the treatments did not have long term effects on fish survival rate.

Fish growth

The 10 hours immersion treatment with a JLP dose of 0.25 mg L^{-1} had the highest value in terms of weight and body length additions (**Figure 1**). No significant differences were observed between treatments based on the body length results graph. However, the 0.25 mg L^{-1} JLP treatment differed in terms of body weight as shown in the body weight graph compared to other treatments. Juvenile growth during the process of sex change was determined by factors such as stocking density, feeding rate, temperature, and environment. The 0.25 mg L^{-1} JLP treatement had a low SR which could be a consequence of high growth. A high stocking density will affect growth, SR, feed efficiency, reproductive performance, and productivity (Diatin 2016). According to Iskandar (2010), tilapia larva (at the age of 4 days) immersion in MT did not significantly affect fish growth compared to control during 60 days. In addition, JLP supplementation by mean of feed, at doses of 37.5 mg kg $fish^{-1}$ day^{-1} and 187.5 mg kg $fish^{-1}$ day^{-1}, during 8 weeks did not affect fish metabolism as the body weight was similar in all treatments (Elisdiana, 2015). Body growth is affected by other factors that are different to hormones that are anabolic.

Table 1. *S. eupterus* larva survival rate (SR) percentage on the 1st and 16th weeks after masculinization treatments.

Observation on week	Masculinization treatments	Immersion duration	
		5 h	10 h
1	P0 (JLP 0 mg L^{-1})	$99.00 \pm 1.00^{a(1)}$	$99.00 \pm 1.00^{a(1)}$
	P1 (JLP 0.125 mg L^{-1})	$97.67 \pm 1.53^{a(1)}$	$97.67 \pm 0.58^{a(1)}$
	P2 (JLP 0.25 mg L^{-1})	$92.33 \pm 3.06^{b(2)}$	$49.67 \pm 21.22^{a(2)}$
	MT 2 mg L^{-1}	$97.67 \pm 1.53^{a(1)}$	$93.00 \pm 2.64^{a(1)}$
16	P0 (JLP 0 mg L^{-1})	$94.67 \pm 1.53^{a(1)}$	$94.67 \pm 1.53^{a(1)}$
	P1 (JLP 0.125 mg L^{-1})	$96.67 \pm 1.53^{a(1)}$	$95.33 \pm 0.58^{a(1)}$
	P2 (JLP 0.25 mg L^{-1})	$86.33 \pm 2.09^{b(2)}$	$46.33 \pm 22.37^{a(2)}$
	MT 2 mg L^{-1}	$94.00 \pm 2.00^{a(1)}$	$88.33 \pm 6.66^{a(1)}$

Description: Different superscript letter following the mean values within the same columns indicate significant differences ($P < 0.05$) between doses among treatments.

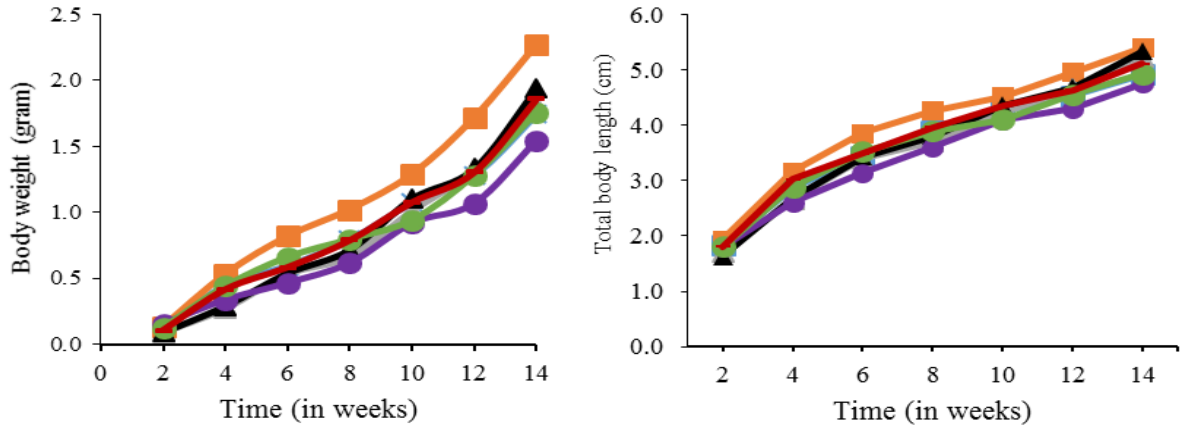


Figure 1. Synodontis fish growth after masculinization treatments with 0.25 mg L^{-1} JLP immersion for 5 h (■),0.25 mg L^{-1} JLP immersion for 10 h (■), 0.125 mg L^{-1} JLP immersion for 5 h (▲), 0.125 mg L^{-1} JLP immersion for 10 h (▲),2 mg L^{-1} MT immersion for 5 h (●),2 mg L^{-1} MT immersion for 10 h (●), control immersion for 5 h (—) and control immersion for 10 h (—).

Gender percentage

Synodontis fish gender at the age of 4 months showed the lowest intersex (60 %) among treatments and male percentage ranged between 25 and 40 %, while female percentage was just 1 % in the MT treatment (**Figure 2**). Immersion duration did not show significant differences among treatments.

The gender percentage of Synodontis fish (age of 4 months old) was dominated by intersex and the MT treatment resulted in 1 % female. MT was believed to experience aromatation in changing androgen into estrogen leading to female individu (Zairin 2002). The total intersex fish was above 50 % in all treatments. Synodontis fish is classified in the order of Siluriformes, which possess a germinal cell development in form of *gonochoristic* that is mainly affected by environmental factors (temperature) during its development (Devlin and Nagahama 2002). *Gonochoristic* order fish will develop in becoming only male or female and the fish will have the same gender in its life cycle. Some fish of *gonochoristic* order experienced a period where all their gonads begin with intersexual stage in order to become (afterward) testes or ovary, for instance marine fish such as (*Cheimerius nufar*) (Coetzee 1983) and (*Gramma loreto*) (Asoh and Shapiro 1997). According to Piferrer *et al.* (2005), European marine bass fish (*Dicentrarchus labrax*) oocyte appears in an intra-testicular form in the gonad, which indicated intersexuality. In intersex gonads, the fish is still unstable and the fish gender could be male or female, which is observed in the percentage of intersex fish that decreases as the fish age.

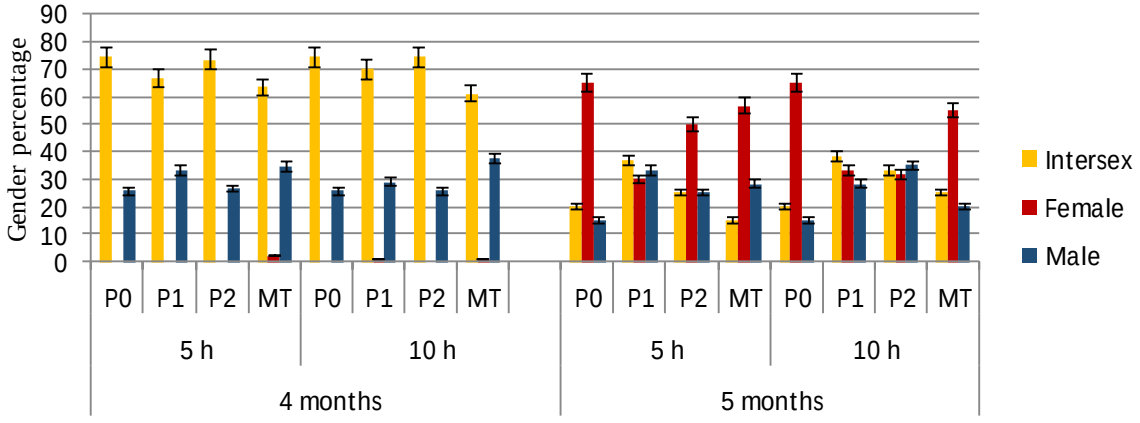


Figure 2. Intersex fish percentage, male and female *S. eupterus* at ages of 4 and 5 months old after masculinization treamnets.

Table 2. Percentage of intersex, male and female of *S. eupterus* at 4 months old and 5 months after treatments.

Observation in month	Masculinization treatments	Percentage		
		Intersex	Male	Female
4	P0 (JLP 0 mg L^{-1})	74.44 ± 4.55^{b}	25.56 ± 4.55^{a}	0.00 ± 0.00
	P1 (JLP 0.125 mg L^{-1})	68.33 ± 8.62^{ab}	31.11 ± 7.79^{ab}	0.56 ± 0.34
	P2 (JLP 0.25 mg L^{-1})	73.89 ± 2.51^{b}	26.11 ± 2.51^{a}	0.00 ± 0.00
	MT 2 mg L^{-1}	62.22 ± 5.02^{a}	36.11 ± 6.47^{b}	1.67 ± 0.56
5	P0 (JLP 0 mg L^{-1})	20 ± 4.47^{a}	15.00 ± 0.00^{a}	65.00 ± 4.47^{b}
	P1 (JLP 0.125 mg L^{-1})	37.50 ± 5.24^{b}	30.83 ± 3.76^{b}	31.67 ± 5.16^{a}
	P2 (JLP 0.25 mg L^{-1})	29.17 ± 8.61^{b}	30.00 ± 8.37^{b}	40.83 ± 13.93^{a}
	MT 2 mg L^{-1}	20 ± 8.94^{a}	24.17 ± 9.17^{b}	55.83 ± 9.70^{b}

Description: Different superscript letter following the mean values within the same columns indicate significant differences (P < 0.05) between masculinization treatments.

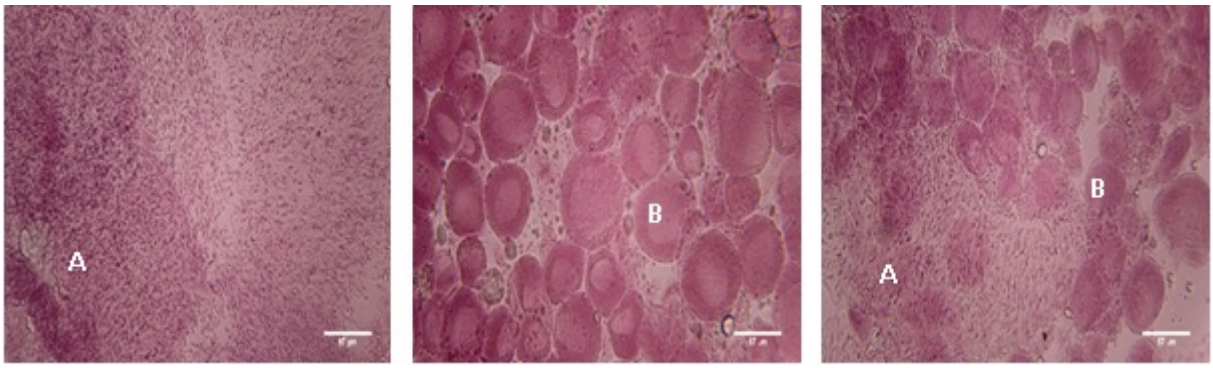
In the fish of 4 months old, the MT treatment resulted in the highest male percentage (*p<0.05*) being 36 % compared to control (P0) and JLP 0.25 mg L^{-1} (P2), but not significantly different to JLP 0.125 mg L^{-1} (P1). The lowest intersex percentage was observed in the MT treatment (*p<0.05*) compared to control (P0) and P2, but was not significantly different to P1 (**Table 2**). While at the age of 5 months old, the intersex percentage significantly decreased up to 20 %, female fish increased to 30 % in P1 and the highest was 65 % in P0 with total male about 15 % in P0 up to 30 % in P1 and P2, while 24 % in MT. The immersion treatments with MT, 0.125 mg L^{-1} and 0.25 mg L^{-1} JLP resulted in higher male percentage compared to control (*p<0.05*). Intersex fish percentage was lower in control and MT treatment treatment and significantly different compared to 0.125 mg L^{-1} and 0.25 mg L^{-1} JLP.

Synodontis fish at the age of 5 months had a higher percentage of female compared to male in control treatments of natural rearing condition at percentages of 65 % female and 15 % male. The mentioned condition is in line with the results of the present research, where the male percentage was lower than that of the female. The percentage was observed to be higher in MT, 0.25 mg L^{-1} and 0.125 mg L^{-1} JLP compared to control.

The high percentage of male at the age of 5 months was assumed to be in relation with the supplementation MT and JLP that contain androgenic through immersion. The androgen content of MT and JCE affected the hormonal system of the fish in order to produce male fish. Hormone supplementation for changing fish gender is aimed at affecting the hormone balance in blood, where during the sex differentiation, a specific individual will become male or female with an extraneous introduction (Rougeot *et al.* 2002). In the present study, the male synodontis percentage at the age of 5 months did not significantly different JLP treatments (doses of 0.25 mg L^{-1} and 0.125 mg L^{-1}) and the MT treatment (2 mg L^{-1}), indicating that JLP extract can substitute MT.

Both 0.125 mg L^{-1} and 0.25 mg L^{-1} JLP were not observed to decrease the intersex percentage and were significantly different compared to control and MT treatments at the age of 5 months. The high intersex percentage in JLP treatment was believed to be due to a low JLP extract which contained phytoteroid. According to Van Der Kraak (1998), the receptor affinity power from plant phytosteroid was lower compared to that of animals. The dose of the supplemented steroid should not be high, since it can put pressure on the formation of the gonads with paradox effects such as low growth and high mortality. According to Zairin (2002) intersex fish derived from anomalies during gender formation due to hormone dose, the immersion duration or a treatment that is not suitable. The sex of the fish could be determined by two factors, genetic and environment (Arfah and Carman, 2008). One of the environmental factors that strongly affects the fish sex is temperature. In the present study, the rearing temperature was 25-27 °C, which was higher compared to the optimal one (22-26 °C) for rearing according to literature (Alderton 2008). Temperature is referred to as a parameter that affects the sex ratio of fish when they start growing at the beginning of their life cycle (Zairin, 2002). According to Goto-Kazeto *et.* al (2006), in general, a high temperature would lead to more male fish in terms of sex ratio, while a low temperature will lead to producing more female fish.

S. eupterus gonad at the age of 4 months was observed using asetocarmin method under microscope. The observation was performed on 30 fish (in each treatment) (**Figure 3**).



1 2 3

Figure 3. Synodontis (4 months old) gonad observation using asetocarmin method 1. male 2. female 3. Intersex with a magnification of 40x. Scale bar of 87 µm. Will become male cell (A), female cell (B).

3. Conclusion

Synodontis fish masculinization at the stage of larva using long Jawa chili extract through immersion with 0.125 mg L^{-1} and 0.25 mg L^{-1} doses can increase twice the percentage of male fish compared to control. Javanese long pepper extract affected the survival rate of fish and the 0.125 mg L^{-1} treatment resulted in the highest survival rate, being 97.67 %. An increase in long Jawa chili extract dose can decrease fish survival rate.

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