The optimum dose and period of synthetic hormones 17-α methyltestosterone during fry stage on masculinization of Siamese fighting fish (*Betta splendens*)

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Abstract - The objective in study aims to develop the production of a mono-sex population of all male Siamese fighting fish (*Betta splendens*) by using 17α-methyltestosterone (17α-MT). Five treatments contained in a control of, MT-20, MT-40, MT-60 and MT-100 in triplicates. The study for two periods, supplemented diet with 17α-MT experimental diet for 30 days (4 -34 days after hatching (dah), Period-I) and for 45 days (4 - 49 dah, Period-II). Experiments were set up using completely randomized design (CRD). The control group diet without the use of 17α-MT. The remaining groups MT-20, MT-40, MT-60 and MT-100 were fed with feed containing 20, 40, 60 and 100 mg kg^{-1} of 17α -MT. Four days old fry were stocked at the rate of 100 fry per tank (Period-I and Period-II). The fry fish with oral administration at first feeding was successfully carried out at four dah. Fish were fed with the experimental diet for two periods (Period-I and Period-II). Gonadal characteristic from Period-I and Period-II reared *B. splendens* for sex reversal was observed. The highest male population was produced from MT - 60 and MT - 100 mg kg^{-1} in Period-II. Also demonstrated, there were no significant differences in the survival rates among the experimental groups. In conclusion, the masculinizations of all males were produced from Period-II treatment group 60 and 100 mg kg^{-1} of 17α-MT successfully produced all-male stocks in Siamese fighting fish.

Keywords: *Betta splendens*, Siamese fighting fish, 17 alpha methyltestosterone, sex reversal

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1. Introduction

Siam fighting fish (*Betta splendens*, Regan, 1910) is an ornamental fish, originating in Southeast Asia and is cultured worldwide, especially in Thailand's Nakhon Pathom province, where it is known as the Siamese fighting fish due to the aggressive behaviour between the males (Giannecchini *et al*., 2012). It is reared in captivity worldwide as an aquarium fish for its favorable characteristics, such as a broad variety of colors, long and wide fins, and the presence of an accessory respiratory organ that allows it to be kept in small tanks with no aeration (Zuanon *et al*., 2009). Betta fish, have semantic differences between males and females. Male fish have more tempting body colors and shapes than females. This causes the selling value and market demand for males to be higher than that of females (Kavumpurath & Pandian, 1994; Sermwatanakul, 2019). Therefore, in order to obtain enough male fish, masculinization procedures are required.

Currently, environmental factors affect the sex ratio of many fish species (Baroiller *et al*., 2009). Due to the variability of the current that affects betta fish culture, such as climate change, and the abundance of natural foods changes. It has a widespread impact on commercial betta production (Puello-Cruz *et al*., 2010). As a result, the production of betta fish in some periods is not stable. This affects the ratio of betta fish sex change (from the original sex ratio to 1:1), which according to inquiries of betta farm operators; it was found that most of the culture has a relatively low proportion of male betta fish. Nowadays, the use of synthetic hormones in fish sex conversion is widely popular among economic fisheries. They are popular as transgender fish to increase the value of commercial production (Taranger *et al*., 2010).

The synthetic steroid 17α-methyltestosterone (17α-MT) has been popular in commercial used, applied by oral and/or immersion treatments in fish farming for sex reversal of fish to all male populations (Bardhan *et al*., 2021). The 17α-MT could be used with fish at all stages. It is a widely used hormone. It disintegrates in a short time (Shrestha, 2003). Commonly, used as a feed additive in commercial farms by ornamental Aquaculturists (Vinarukwong *et al*., 2018). Investigate the effect of 17α-MT concentration and frequency on Guppy (*Poecillia reticulate*) sex inversion by immersion 400 μg l^{-1}, the percentage of maximum males were observed (Mekdaeng, 2015). Crossbreeding Cichlids with dietary 17α-MT at 40 and 60 mg kg^{-1} of feed for 21 days would result in increased percentages of males (above 70%) of Flower Horn (*Amphilophus citrinelles* x *A. trimaculatus*). Some researches effort to obtain all males in betta fish can be done by using hormonal sex reversal at the first day of feeding (Kavumpurath & Pandian, 1994). Moreover, immersion with *Moina macrocopa* 200 ppm by fluoxymesterone for 20 minutes and fed 14 days. It can produce all males, which can be differentiated by color and fin (Tangtrongpairot *et al*., 1988). By the way, different results were reported depending on the dose of 17α-MT, administration duration time initiated as well as environment conditions (Piferrer, 2001).

This study aimed to evaluate the effectiveness of oral administration by first feeding with a powder feed at the ideal concentration and duration. To introduce a new appropriate bioenvironmental method in *B. splendens* on sex reversal and survival rate to produce more males (compared to females) remains effective, as well as a local education, those that have a high culture volume of fighting fish may be able to develop a culture technique to create appropriateness in the next culture model.

2. Material and methods

2.1 Preparing broodstock and fry production

The Muban Chombueng Rajabhat University Animal Care and Use Committee's Ethics Committee accepted all protocols pertaining to the study's experimental animals. (approval no. AS6607003). The broodstock (6 months old) Siamese fighting fish (*B. splendens*) were obtained from commercial farms (20 pairs of broodstock) and maintained at Muban Chombueng Rajabhat University (Ratchaburi, Thailand). Broodstock were maintained individually in a plastic bottle (800 ml) with a natural light/dark cycle. The fish were fed a commercial feed containing 32% protein and 4% fat) ad libitum twice a day at 10.00 and 16.00. The cleaned plastic bowls were used as the pond to build bubble nests. In each mating pond (in total 20 bowls) one male (average body weight 1.90 g) and one female (average body weight 2.05 g) were cultured. Before mating, the female fish were put in one aquarium and the males were given a partition for 12 hours (hrs) before mating. After 12 hrs from mating the fertilized eggs were obtained, 24 hrs post fertilization, and the broodstock was removed adapt from Lichak *et al*. (2022). Fish larvae were transferred to a concrete pond (diameter 0.80 m depth 0.5 m) until three days after hatching (dah) which at this period the digestive tract of *B. splendens* larvae was fully developed (Silva *et al*., 2016).

2.2 Experimental design, experimental diet, sex reversal and fish culture

Three thousand *B. splendens* fry of uniform size (approximately 2 mm) were equally distributed in five treatment groups with each of three replicates in a glass aquarium of 35 L capacity in two periods (period-I, period-II). The experiments were performed using a completely randomized design (CRD) with five treatment hormone concentrations; each replicated three times (five treatments, three replicated). Experimental diets were prepared by incorporating 17α-MT (Sigma-Aldrich Chemical Co., St Louis, MO, USA) into a commercial diet containing 50% protein and 10% fat. The five treatments were as basal diet (control), 20 mg kg^{-1} (MT - 20), 40 mg kg^{-1} (MT - 40), 60 mg kg^{-1} (MT - 60), and 100 mg kg^{-1} follows; (MT - 100), 17α-MT supplemented diets. To prepare the hormonal treatment diets, 17α-MT (20, 40, 60 and 100 mg) was dissolved in 95% ethanol (200 mL) and sprayed on the commercial powder feed. The identical process was used to make the control feed, but no 17α-MT was added. The feed was treated with the same amount of ethanol applied from Kumkhong *et al*. (2020). After the dispersal of ethanol for 12 hrs in the dark at room temperature, all experimental diets were kept in a refrigerator at 4 °C, covered by dark plastic bags until use.

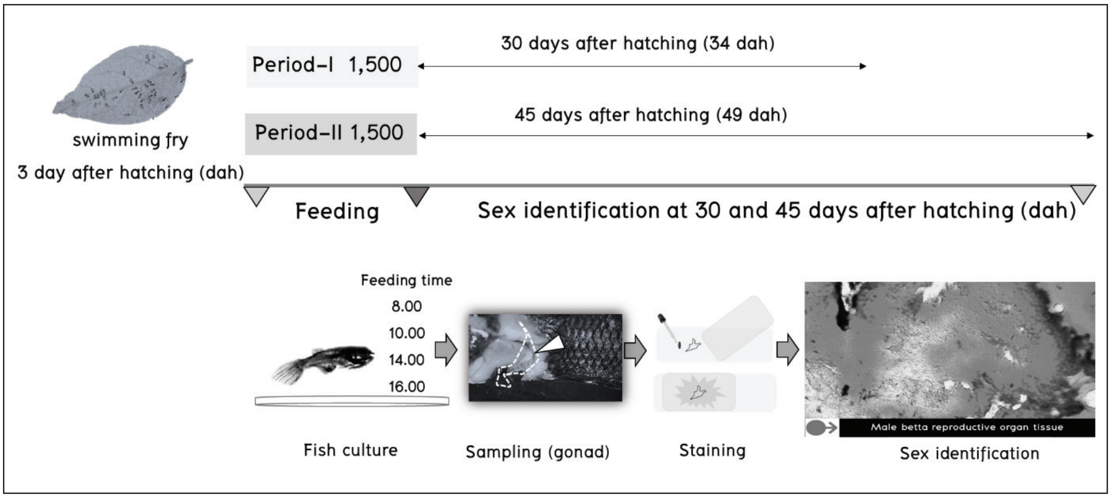

Figure 1. Experimental plan of 17-α Methyltestosterone during fry stage on masculinization of Siamese fighting fish (*B. splendens*). Fry at 4 days after hatching (dah) were fed with each experimental diet for 30 days (4 -34 dah, Period-I) and for 45 days (4 -49 dah, Period-II). Subsequently, at the end of the experiment, trial fish (Period-I and Period-II) (30 fish/replication) were sampled for sex identification. Afterwards, fish sampling was carried out.

The experimental design fry culture used in this investigation is shown in Figure 1. Thirty ponds (circular ponds with 0.6 m diameters and 0.3 m of water) with a 12-hour/12-hour light-dark cycle used in the experiment. After being transferred, three dah fry (n = 3,000) were divided into groups of 100 fry at random in each of the fifteen ponds. At 4 dah (about 2 mm), the fish were fed four times a day (10.00, 12.00, 14.00, and 16.00; adapted from Kumkhong *et al.* (2020)) with 17α-MT enriched meals (commercial feed 50% protein, 10% fat) until they were satiated. Up to the conclusion of the experimental periods, which were 30 days after hatching (Period-I) and 45 days after hatching (Period-II), the fish were fed diets supplemented with hormones to sustain their growth. Every three days, waste materials were replaced and siphoned off. The results of the daily measurements of the water temperature and air temperature were 27.73 ± 0.67 °C and 33.19 ± 1.80 °C, respectively. The weekly evaluations of pH, dissolved oxygen (Hanna; HI98194), ammonia NH_3/NH_4 (ppm), and nitrite NO_2^- (ppm) were found to be within acceptable ranges of 8.00 - 8.50, 3.10 - 3.41 mg L^{-1}, 0.17 ± 0.06 ppm, and 0.15 ± 0.07 ppm, respectively.

3. Fish sampling and experimental protocol

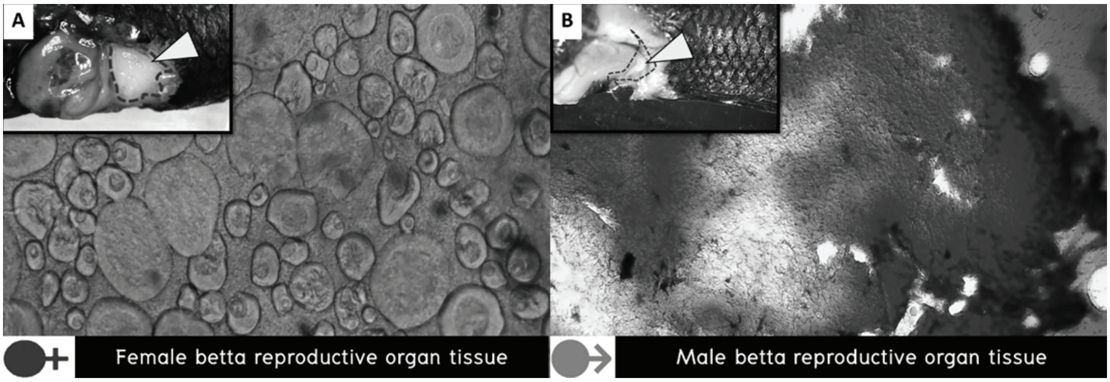

Figure 2. Schematic representation of the characteristics in female gonad differentiation in females (A) and males (B) of the Siamese fighting fish (*B. splendens*). Male gonads were found in the presence of spermatozoa which appeared much smaller than the egg cells (the arrow) and had a large number of what looked like evenly spread spots. In this protocol, both gonads were examined microscopically after being stained with 0.2% crystal violet solution (magnification of 40 X).

To determine the sex ratio, both experimental periods were euthanized with 10 % clove oil (Kumkhong *et al*., 2020), and injected with 0.10 ml of 5% acetic acid in the abdomen. The gonads were removed for sex identification. The squash method, which originated from Wassermann & Afonso (2002), was used to determine the sex ratio in each group using a microscope to identify the gonadal tissue. All gonads were examined microscopically after being stained with 0.2% crystal violet. For the purpose of determining the gonadal sex, all gonads were examined using a compound microscope at 40 x and 100 x magnification. Sex was judged and recorded as male or female by gonadal tissue showing oocyte or spermatogonial cells as refered to in Figure 2 (Pattiasina *et al*., 2021).

3.1 Measuring parameters

The measured parameters were the sex ratio and survival rate formation. Identification of sex in betta fish is carried out by characteristics in gonad differentiation (female or male) observations when the fish larvae were fed until the end of both experimental periods. According to Pattiasina *et al*. (2021) the male-sex ratio can be calculated using the

following formulation:

Sex ratio of male (%) = (Number of males /total fish sample) x 100

Sex ratio of female (%) = 100 - % of Males

Survival rate (%) = (Number of fish at the end of rearing / Number of initial fish stocked) x 100

3.2 Data analysis

Alterations in male sex ratio percentage of Siamese fighting fish were analyzed between treatments by one-way analysis of variance (ANOVA). To evaluate differences between the Period-I and Period-II, an independent t-test was performed. The sex ratio within each group was tested to compare (male and female ratio 1:1) by the chi-square test. The variation was examined as the male to female ratio. The orthogonal contrasts method was used to investigate a discrepancy in male percentage means. Using the R application, all of the study's data were examined using a 95% confidence level. (R version 4.0.3 (2020-10-10)).

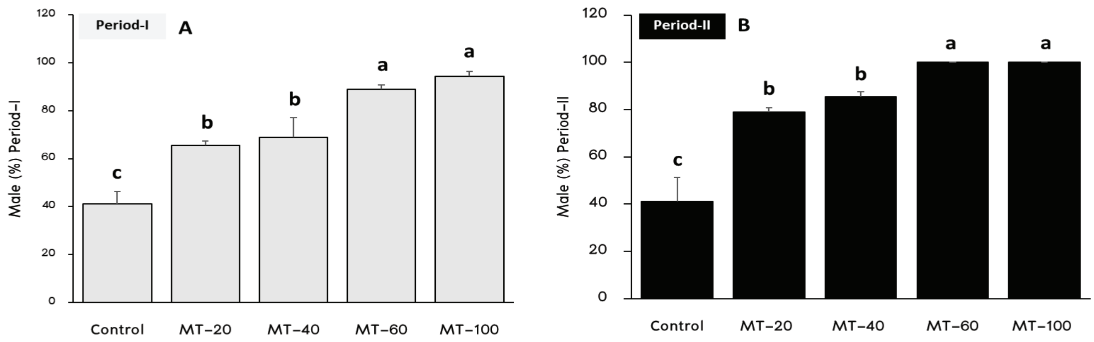

Figure 3. Percentage of male Siamese fighting fish (*B. splendens*) supplemented 17α-MT in diet during Period-I (A) and Period-II (B) on male sex ratios. Data are presented as the mean ± standard deviation (SD). Number of fish = 90 per hormonal treatment, sampled at Period-I and Period-II (30 fish/replication; total = 3 replicates). Means with different superscripts showed significant difference from each other ($P < 0.05$).

4. Result

This study demonstrated the success of using hormone compounds in betta culture to increase the number of male Siamese fighting fish. Studies were conducted to determine appropriate 17 α -MT hormone concentrations. The duration of hormone administration that showed a change in the amount of male betta fish was increased in the male betta population compared to the control group (P<0.05) (Figure 3 A, B). Dietary supplemented 17 α -MT since 20 -100 mg kg^{-1} of two periods (Period-I and Period-II) was increased present male betta by 65.56 % -100.00 % (P<0.05) in Table1. The Period-I of hormone resulted in an increase in the rate of male betta fish by more than 80 %, especially hormone concentrations of 60 and 100 mg kg^{-1} (Table 1). Nonetheless, in Period-I (4 - 34 days after hatching), it is not possible to change the sex of all males in betta (Figure 3A and Table 1).

In this study increasing the duration time of feeding in Period-II (4 - 49 day after hatching) feeding diet supplemented 17α-MT (20 -100 mg kg^{-1}) in the diet had a positive effect on male sex present in betta (Figure 3B and Table 1), whereas the 17α-MT initial level from 20 mg kg^{-1} had the effect on sex ratio of betta fish as during the feeding time in Period-I (4 -34 day after hatching) (P<0.05). However, the level at which all male in betta fish can be (P<0.05) at 60 and 100 mg kg^{-1} (100 % male sex in betta) as shown in Figure 3B and Table 1.

Table 1. Effects of 17α-MT supplemented in diet during Period-I and Period-II on sex ratios of Siamese fighting fish (*B. splendens*)

	Control	**MT - 20 (mg Kg^{-1})**	**MT - 40 (mg Kg^{-1})**	**MT - 60 (mg Kg^{-1})**	**MT - 100 (mg Kg^{-1})**
Period-I (4 -34 days after hatching)					
No. of male	37.00	59.00	62.00	80.00	85.00
Male (%)	41.11	65.56	68.89	88.89	94.44
No. of female	53.00	31.00	28.00	10.00	5.00
Female (%)	58.89	34.44	31.11	11.11	5.56
Sex ratio M:F	1:1.43	1:0.53	1:0.45	1:0.13	1:0.06
*X^2	2.1209	9.6845	14.273	60.497	78.997
P-value	0.145	0.002	0.001	<0.001	<0.001
Period-II (4 -49 days after hatching)					
No. of male	37.00	71.00	77.00	90.00	90.00
Male (%)	41.11	78.89	85.56	100.00	100.00
No. of female	53.00	19.00	13.00	0.00	0.00
Female (%)	58.89	21.11	14.44	0.00	0.00
Sex ratio M:F	1:1.43	1:0.27	1:0.17	1:0.00	1:0.00
*X^2	3.1613	33.385	50.581	100	100
P-value	0.0754	<0.001	<0.001	<0.001	<0.001

Abbreviations: MT = 17-α Methyltestosterone; MT-20 = dietary MT supplementation at 20 mg kg^{-1}; MT-40 = dietary MT supplementation at 40 mg kg^{-1}; MT-60 = dietary MT supplementation at 60 mg kg^{-1}; MT-100 = dietary MT supplementation at 100 mg kg^{-1}. Number of fish = 90 per hormonal treatment, sampled at Period-I (4 -34 days after hatching) and Period-II (4 - 49 days after hatching) (30 fish/replication; total = 3 replicates). * Using Chi-Square test (X^2), the calculated X^2 was significantly different from the expected X^2 (1:1 ratio).

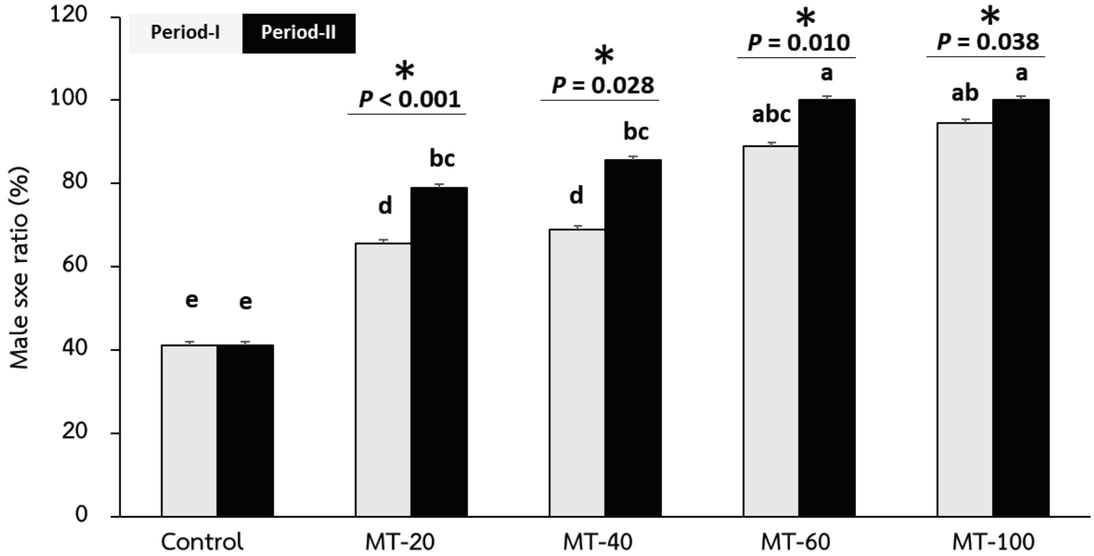

Figure 4. Male sex ratio (%) of the Siamese fighting fish (*B. splendens*) fed supplemented 17α-MT in the diet. Data are presented as the mean ± standard deviation (SD). Number of fish = 90 per hormonal treatment, sampled at Period-I (4 -34 days after hatching) and Period-II (4 -49 days after hatching) (30 fish/replication; total = 3 replicates). Means with different superscripts shown differ significantly from each other ($P < 0.05$). The asterisk (*) in the bar graph indicates a significant difference ($P < 0.05$) between Period-I (4 -34 days after hatching) and Period-II (4 -49 days after hatching).

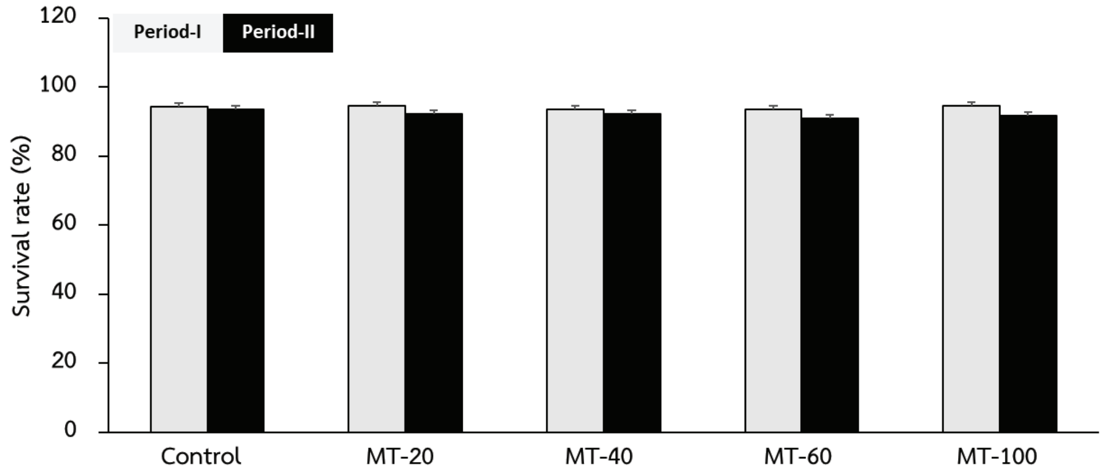

Figure 5. Percentage of survival rate of the Siamese fighting fish (*B. splendens*) fed supplemented 17α-MT in the diet. Data are presented as the mean ± standard deviation (SD). Number of fish = 90 per hormonal treatment, sampled at Period-I (4 -34 days after feeding) and Period-II (4 -49 days after feeding) (30 fish/replication; total = 3 replicates)

Moreover, supplemented 17α-MT in the diet and feeding in Period-II at hormone concentrations of 60 and 100 mg kg^{-1} had the highest effect on transsexuality rates (all male) by feeding hormone-infused ($P<0.05$) (Figure 4). The results of the test at the right period of time showed the duration of hormone in Period-II administration had the highest positive effect on the rate of sex reversal ($P<0.05$) (Figure 4). Finally, studies by hormone-supplemented in diet and feeding at first meal administrated had a detrimental effect on fish, finding that survival rates of all hormone concentrations administrated and period of the time had no effect on survival rates ($P>0.05$) Figure 5.

5. Discussion

Successful feminization has been proven to depend on a number of variables, hormones used, their dosage, and the hormonal therapy used with oral hormonal administration or immersion stage of fish (egg or fry stage), and the duration of treatment. For instance, oral hormonal administration during the fry stage is more effective (Singh *et al*., 2019; Soumokil *et al*., 2020; Kipouros *et al*., 2011; Wahbi *et al*., 2010; Arslan *et al*., 2009; James & Sampath, 2006). Early feeding supplemented with 17 α -MT in the diet has been shown to improve male sex reversal in ornamental fish. The study in Green Swordtails (*Xiphophorus hellerii*) fed 17 α -MT treated feed at 3% or 5% body weight for 28 days resulted in 100% masculinization, with the development of male secondary sexual characteristics, specifically the growth of the sword extension (Yanong *et al*., 2006). The experiment with fry of Siamese fighting fish orally administered with 17α-MT (via inert feed) in four concentrations (1, 2, 3 and 4 mg kg^{-1} of hormone in feed) for eight weeks. The findings demonstrated that the 3 and 4 mg kg^{-1} doses effectively masculinized the progeny, and the extended period of enriched *Artemia* sp. *nauplii* raised the offspring survival rate. (Kipouros *et al*., 2011). Also in Red Swordtail *Xiphophorus helleri* and Siamese fighting fish; *B. splendens* were investigated. *B.splendens* treated with 20, 40, 60 mg kg^{-1} produced 100% male population on day 84, 56 and 56, respectively, while *X. helleri* treated with 40 and 60 mg kg^{-1} produced a 100% male population on day 70 and 56 (James & Sampath, 2006). In the present study, dietary supplementation with 17 α -MT during the fry stage successfully produced a male population of Siam fighting fish (*B. splendens*). Long dietary supplementation of 17 α -MT at 60 and 100 mg kg^{-1} for 45 days after hatching (Period-II) successfully produced 100% males, while short dietary supplementation at the same dosage for 30 days after feeding (Period-I) was not efficient (in this study), suggesting Period-II as the masculinizing treatment in *B. Spendens*. The optimal duration for masculinizing treatment in the characteristics study of the gonads at Period-II (4 -49 days after hatching) indicated that the gonad shad developed into testes. Exogenous 17 α -MT added to the diet promoted the development of a male population; however, the dose of 17 α -MT added to the diet and the proportion of males vary depending on the species, stage of the fish, hormone levels, and the length of treatment (Piferrer, 2001).

Our study demonstrated the survival rates among the experimental groups and duration time in our study did not differ significantly, indicating that dietary supplementation with 17 α -MT did not negatively affect masculinization of

Siamese fighting fish (*B. splendens*). Other fish species (Spotted snakehead, *Channa punctatus*; White carp, *Cirrhinus mrigala*) have reported experiencing similar observations (Muniasamy *et al*., 2019; Pattiasina *et al*., 2021). To produce mono-sex cultures, hormonal sex reversal has been done on a variety of fish species, each with a different level of public appeal and viability for business. Meaningful is the fact that since the experimental fish were raised, this experiment is also looking for the appropriate level of hormone concentration and administration period.

6. Conclusion

Based on the results of this study, it can be concluded that masculinization through oral administration at first feeding in betta fish fry was successfully carried out at four days after hatching. The increase in the percentage of male sex ratio of *B. splendens* was influenced by the time and 17α-MT concentration. Supplemented 17α-MT at 60 and 100 mg kg^{-1} for Period-II had the highest male sex ratio values (100% male). It can be concluded that masculinization was successfully carried out in Siam fighting fish.

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