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Research Article
Effect of Larval Ages and Honey Treatment on the Sex Reversal of Betta Fish (*Betta splendens*)

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Abstract
Background and Objective: Betta fish is one of the most popular ornamental fish because of their attractive body shape, especially for males. The fish development usually results in a similar number of males and females or an even higher number of females. This study aimed to determine the effect of various concentrations of honey and the age of larvae on the sex ratio of Betta fish. **Materials and Methods:** This study was conducted using a completely randomized design with two variables; the concentrations of honey and the ages of Betta fish larvae in 3 replications. There are 45 groups in total with 20 larvae in each group. The levels of honey concentration were 0 (control), 10, 20, 30 and 40 mL/L and the levels of larval ages were 3, 4 and 5 days old. All groups were immersed for 10 hrs in the honey mix and then maintained for 4 months. Sex ratio calculation was performed after 3 months. **Results:** The both factors have a significant effect on male sex formation. The highest number of males was produced in the honey concentration of 40 mL/L. A significant difference in the number of males was shown in the larval group of ages 3 and 4 days old but not in the groups of 5 days old. **Conclusion:** Honey treatment and the larval age affect the formation of male Betta fish.

Key words: Betta fish, honey concentration, larval ages, sex ratio, sex reversal

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The ornamental fish market rises around 70% of the global capital, with around 90% of these animals being produced in captivity[1,2]. In the last 20 years, Indonesia has participated as one of the global ornamental fish exporter[3]. One of the main commodities of ornamental fish that has the potential to be developed in Indonesia is Betta fish (*Betta splendens*)[4]. Betta fish is very popular among ornamental fish farmers around the world and its cultivation has brought huge economic benefits to the farmers[5,6]. Betta fish have distinctively striking characteristics, with the superiority of the males due to their colorful bodies with longer tails and fins[7,8]. Therefore, male fish are more costly with 4-10 times higher prices as compared with the females[1-4].

The problem in Betta fish cultivation is the low number of male fries obtained in each spawning process when compared to that of female fries[9]. As reported by de Fiuza França *et al.*[1] and Iryanto *et al.*[9] in every spawning time only of 40% of males were obtained while the remaining 60% were females[1,9]. Previous studies confirming an incomplete genotypic sex determination in Betta fish[10,11] also found a minor proportion of homogametic males, suggesting the influence of environmental factors in sex determination in Betta fish.

Sex reversal is a genetic engineering reversing the genital development from female to male (masculinization) or male to female (feminization)[12-14]. Phenotypic sex reversal may occur simultaneously in the same individual[14,15]. The manipulation of sex reversal in fish farming is a well-known practice to obtain animals with sex owning higher commercial value in the market, including *Betta* sp.[1]. The most popular masculinization procedure is the administration of steroid hormones, which regulate sex formation by activating sex hormones in both male and female organisms during gonadal differentiation in undifferentiated fish[16,17]. Several investigations have shown that dietary treatment of synthetic steroid hormones can masculinize Siamese-fighting fish[18,19]. However, synthetic steroid hormones are potentially harmful to the environment and public health[20].

Administration of an aromatase inhibitor may also masculinize fish in aquaculture. Aromatase has a role in sex differentiation and ovarian development by transforming androgens into estrogens[21,22]. Thus, blocking the aromatase enzyme can promote androgen production while inhibiting estrogen. A recent study reported that administration of fadrozole, an aromatase inhibitor, leads to masculinization of Japanese flounder (*Paralichthys olivaceus*) by increasing androgen, which is responsible for testicular differentiation[23,24].

One of the aromatase inhibitors is chrysin, a kind of flavonoid found in natural honey[25,26]. In addition to its environmental safety, natural honey contains high levels of potassium which regulates the circulation of testosterone and controls the effect of androgens, thus operating as a driver of fish sex differentiation[4,27]. In earlier research, the use of honey as an agent for masculinizing some fish species has been successfully carried out[4,5,28-32]. The use of honey is anticipated to guide the process by which female gonads differentiate into male gonads, hence influencing the morphological traits of the Betta fish offspring.

Factors that influence the success of the masculinization process in fish are the concentration of honey applied and the duration of treatment. The relationship between the concentration of honey and the age of the fish needs to be considered to obtain the expected results. Previous studies have reported the effect of immersing fish larvae in honey solution on increasing the male-fish ratio in several farmed fish species, yet whether the ages of fish larvae treated with the honey solution could affect the sex reversal is still unknown, especially in Betta fish species. Thus, the present study aimed to investigate the effect of immersion of Betta fish larvae of different ages in honey solution on the development of male fish. In addition, the effects on the survival rate of the fish were also evaluated.

MATERIALS AND METHODS

Study area and source of broodstock: This research was carried out from March to October 2020. The 500 mL of honey used in this study was wild forest honey taken from the District of Bontocani, Bone Regency, South Sulawesi, Indonesia. Three pairs of mature Betta fish, males and females, at four months of age, were purchased from the ornamental fish market in Makassar. The research was conducted in Biology Experimental Farm, Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar, Indonesia.

Parent maintenance and spawning: Betta fish brood stock was individually acclimated to laboratory conditions in aquaria of 1 L with dechlorinated tap water (at 25°C, pH 6.5) and fed twice a day with *Daphnia* sp., for 2 weeks. The water was renewed daily and feces and debris were removed by siphoning. The animals remained for 2 months until spawning. Before mating, female and male Betta fish are confirmed to have mature gonads by the age of 6 months. The aquarium, with dimensions of 30×40×12 cm, was used as the mating pond. In each pond, transparent plastic was floated on the surface of the water as a nesting place. A shelter was put in the center of each aquarium. One female was brought inside the shelter, while male fish were placed outside for 24 hrs for acclimatization and release of mating pheromones. After a male spawner formed a bubble nest, the shelter was removed to let the male and female begin mating. The female was then removed from the tank. Oxygenation aided the fertilization of the eggs and the eggs hatched in 24-48 hrs, after which the male spawner was removed. After the yolk sac had been consumed, the 3-5 days post-hatch (dph) larvae were moved to a fresh container for the honey immersion treatment.

Sex reversal experiment: The experiment was conducted using a Completely Randomized Design (CRD) in a factorial pattern. Betta fish larvae of different ages are kept in an aquarium filled with 1.5 L of water with a pH of 6.5. The experiment design consisted of two variables: The concentration of honey in water and the age of larvae. There were 5 groups according to the levels of natural honey concentration, namely A1 (10 mL/L), A2 (20 mL/L), A3 (30 mL/L), A4 (40 mL/L) and A0 without natural honey as a control. In addition, there were 3 groups of larval ages: B1 (5 days old), B2 (4 days old) and B3 (3 days old). There were 15 treatment combinations in triplicate. Every group consisting of 20 Betta fish larvae was treated in an aquarium. All larvae in the treatment groups were immersed in a natural honey solution according to their concentration of treatment for 10 hrs. After the treatment is complete, the larvae are transferred to a rearing container until day 90 to observe the development of sex phenotypically or the appearance of secondary sex characteristics.

Larvae care: During the experiment period, the larvae were fed with live *Daphnia* sp., twice a day. The water was changed every week until the larvae were 1 month old. Afterward, the water was changed every 3 days until the Betta fish was 2 months old. The fish are then transferred into new aquariums with 5 L of water, up to 3 months old. The aquarium water was changed every 3 days, while the fish were fed twice a day. At the end of the third month, the sex ratio of the fish was calculated.

Parameter measurement: Identification of sex in Betta fish was carried out by morphological observations and secondary sex characteristics when the fish larvae were 90 days old. The number of males and females and the survival rate were then measured. According to Maulana *et al.*[33], the male sex ratio can be calculated using the following formulation:

$$\text{MI (\%)} = \frac{\text{mI}}{\text{sI}} \times 100 \qquad (1)$$

Where:
MI = Male sex ratio (%)
mI = Total male fish
sI = Total observed fish

The survival rate is an organism's survival level during the rearing period or experimental activity. The survival rate value is obtained by calculating the difference between the number of organisms that live at the end of maintenance and the number of organisms at the time of stocking, expressed in percent[9]. Based on Effendi *et al.*[34], the survival rate can be calculated with the following formula:

$$\text{SR (\%)} = \frac{\text{Nt}}{\text{No}} \times 100 \qquad (2)$$

Where:
SR = Survival rate (%)
Nt = Total of fish on the final rearing period
No = Total of fish on the initial rearing period

Statistical analysis: The data were analyzed using descriptive statistics and inferential statistical analysis using the F test with $\alpha = 0.05$, followed by the LSD test with $\alpha = 0.05$ using GraphPad Prism 9.

Ethical consideration: No humans were used in this research. All the experiments on animals were in accordance with “Guidelines for the Use of Fishes in Research, 2014”[35].

RESULTS AND DISCUSSION

Survival rate (SR) of Betta fish larvae: The negative impact of materials can be shown by the survival rate. The SR value describes the substance’s suitability as a phytohormone source for the masculinization process. It is found that all concentrations of honey treatment do not affect the survival rate. There was no significant difference between the SR of honey treatment and with control group after 10 hrs of treatment (Fig. 1a-c). The SR was not influenced by the age of the larvae. The SR of larvae in the group of ages 3, 4 and 5 days were not significantly different. The mortality rate of the fish in all groups was below 30%, indicating that the use of honey solution as a sex reversal agent is still relatively safe[7,36].

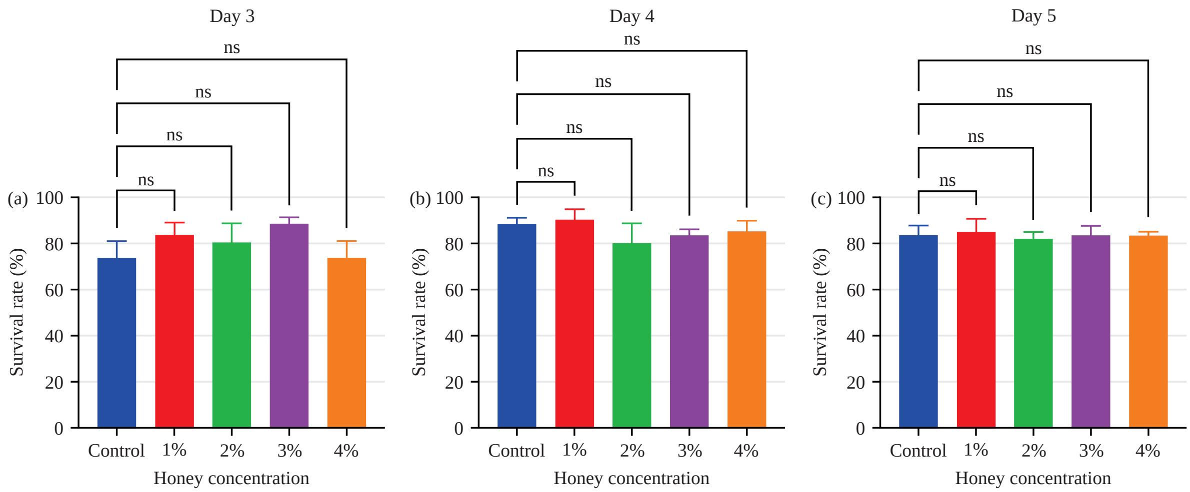

Fig. 1(a-c): Percentage of survival rate of *B. splendens* fed during larvae rearing (90 days)

Data are presented as the Mean±Standard Deviation (SD), number of fish larvae = 20 per treatment or replication, total = 3 replicates and ns: Not statistically significant ($\alpha = 0.05$)

Effect of honey concentration and larva's age on Betta fish sex reversal: Next, the effect of different concentrations of honey immersion evaluated on the sex reversal of Betta fish larvae. The result shows that both honey concentration and age of larvae affect the sex reversal. When honey treatment was applied to larvae of age 4 and 5 days, only 4% honey group showed significantly different numbers of males and females compared to the control group (Fig. 2a-b). However, when the honey treatment was applied to 3-day-old larvae, all concentrations produced a significantly higher number of males compared to the control group, in a stepwise pattern (Fig. 2c).

Effect of different ages of Betta fish larvae on fish sex ratios: Finally, the effect of different ages of Betta fish larvae when treated with honey solution on fish sex ratios was evaluated (Table 1). The percentage of male fish treated for 5 days differs significantly from 3 and 4 days. However, the percentage of male fish in treatment for 3 days is not statistically different from that of four days. This finding suggested that the optimum period to introduce honey is between the ages of 3 and 4 days.

During the sexually labile stage, both internal factors: Hormonal system, chromosomal genes and autosomes and environmental factors: Hormones, other chemicals and rearing medium characteristics, contribute to fish sex differentiation[37]. Sex reversal is a technique that can be used to acquire monosexual offspring, for example, male fish. In this study, to obtain a high male-sex ratio, we immersed different ages of larvae in a honey solution at different concentrations. The present study result demonstrates that the treatment with 3 and 4% honey direct the formation of the male sex superiorly compared to lower honey concentration treatments (1-2%). The same results were observed when the treatment was applied to fish of different ages (3, 4 and 5 days old).

Thus, it is concluded that honey treatment affects the sex ratio of male Betta fish. This conclusion was similar to that of previous studies, which used honey immersion for sex reversal of Betta fish[4,5,9,28], Nile tilapia (*Oreochromis niloticus*)[30], Platy pedang (*Xiphophorus hellerii*)[31], Rainbow fish (*Melanotaenia boesemani*)[32] and Green Swordtail (*Xiphophorus helleri*, Heckel 1848)[29]. The increase in the number of males is probably due to chrysin, a type of flavonoid in honey that can inhibit the aromatase enzyme- an enzyme that reduces the concentration of estrogen. Chrysin will bind permanently to the active side of aromatase thus making the enzyme inactive[30]. The decrease in estrogen will be followed by an increase in the production of testosterone, leading to the differentiation of males[38]. In addition to chrysin, honey also contains potassium that can convert fat into pregnenolone, a hormone that converts estrogen into progesterone[4,27,30]. Every 100 g of honey contains approximately 205-1676 ppm of potassium[39]. The high potassium content of honey administered to cork fish seeds can promote alterations in cholesterol levels in larval tissues to pregnenolone, which is used by the adrenal glands to synthesize steroid hormones[30,40]. This steroid affects the formation of testosterone, so fish that are initially female will develop into male fish[5].

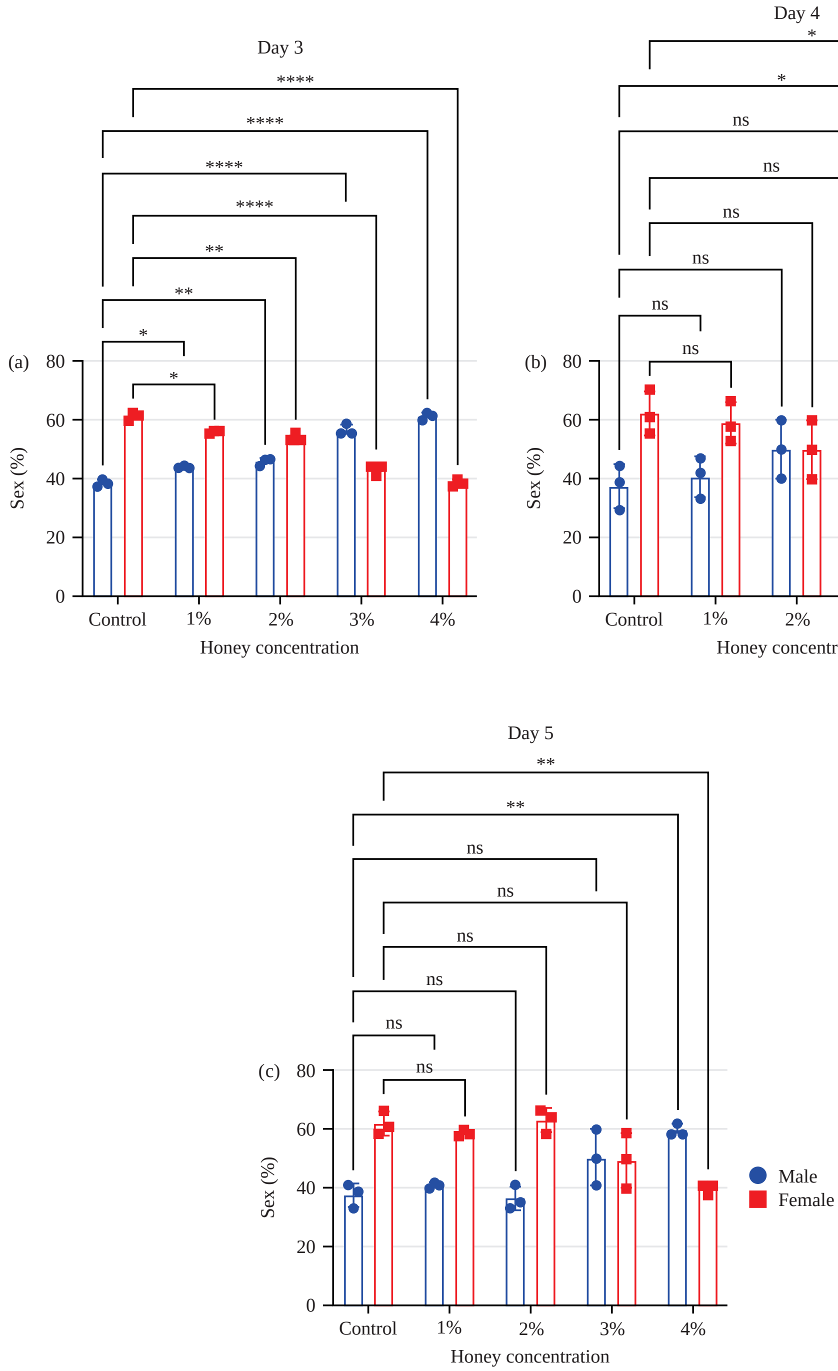

Fig. 2(a-c): Effect of honey concentration and larva's age on the sex reversal of Betta fish

Data are presented as the Mean±Standard Deviation (SD) from triplicate experiments of each group (n = 20 larvae), ns: Not statistically significant, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

Table 1: Effect of different ages of Betta fish larvae on fish sex ratios

Larval age (days)	Mean percentage of male fish	LSD test*
5	45.19	b
4	49.83	a
3	49.31	a

*Value with the same letter(s) in a column are not significantly different at α = 0.05

In this study, it was also found that increasing the concentration of honey used to soak Betta fish larvae affects the number of male fish. These findings are consistent with previous research by Mustaqim *et al.*[29], which found that the higher the concentration of honey utilized, the higher the percentage of male fish obtained, might be due to the increase in chrysin activity. The groups with higher concentrations of honey have higher inhibitory efficacy[30,31,41]. The percentage of male fish obtained from the previous study may be different from this study because of several factors, such as the species of fish used, the type of honey and the age of the larvae.

The present study demonstrates that the treatment of younger larvae results in more males. In the young larvae, the gonads have not been differentiated. The older the larvae, the more advanced the differentiation rate, so that the chances of sex reversal are getting lower. Pattasiana *et al.*[5] and Mulia *et al.*[42] found that the older the fish larvae when masculinization agent is applied, the lower the percentage of male fish obtained. The present study revealed that the best time to introduce honey is 3-4 days after hatching. This could be because fish larvae aged 3-5 days post-hatching still have membranes that are permeable to the honey solution so that the honey solution can diffuse into fish tissues and organs more easily[5,43]. The effect of honey immersion on gonad differentiation is thought to occur when honey diffuses through the body and circulatory system until it reaches the target organs[5,28]. The honey solution enters through the gills and lateral lines[5]. In younger fish larvae, the tissues that make up the body's larvae are thinner, making the process of diffusion easier. In addition, 3-4 days after hatching is an unstable phase, when fish are in the process of determining and directing sex. In this phase, the gonad genotype of the fish has been determined, but its development has not yet occurred, so the administration of the honey solution can influence the development of the gonads to become male.

Finally, one of the important criteria that must be considered when choosing an ideal substance for fish masculinization is its low toxicity. The concentration of the masculinizing agent must be safe for fish. It requires a mortality rate of less than 30% to state that a masculinization agent is safe for fish[7,36]. In the current research, the toxicity of the honey solution used for the masculinization of Betta fish larvae was also evaluated. It was found that the survival rate of fish immersed in honey solutions with concentrations of 1 to 4% was not significantly different from the control group. In addition, the SR value of all groups was above 75%, achieving the safety criteria of a masculinization agent in Betta fish[7,36]. The mortality rate is not different among fish larvae aged 3, 4 and 5 days.

The survival rate of fish larvae is influenced by the concentration and length of immersion time. The appropriate concentration and the right length of immersion time will result in a high percentage of larval survival. Conversely, if the concentration and immersion soaking time are not applied correctly, the larval survival rate will decrease. From the results of this study, it is concluded that 10-40 mL/L are appropriate and acceptable concentrations of honey immersion for the masculinization process in Betta fish. The use of honey concentrations above 4% will have an impact on reducing the survival capacity of fish, as reported by Soumokil *et al.*[4], in which 5-7% of honey causes a decrease in the SR value of between 25-30% in Betta fish. In addition to honey concentration and immersion time, the type and availability of food and the cleanliness of the water used as rearing media also influence the survival of the larvae[4,9]. The high number of survival rates in this study shows that the type and availability of food, as well as the level of cleanliness of the water media used, are suitable for rearing Betta fish.

CONCLUSION

Immersion of Betta fish larvae in a honey solution had a significant impact on increasing the number of male fish. Honey concentrations of 3 and 4% gave the best results in influencing an increase in the ratio of male fish. The age of fish larvae immersed in honey solution influences the increase in the number of male fish obtained. Larvae aged 3 and 4 days after hatching have a higher potential to undergo sexual orientation to become males compared to larvae aged 5 days. Further research is necessary to understand the detailed mechanisms by which the compounds in honey influence sex change in Betta fish.

SIGNIFICANCE STATEMENT

The purpose of this study was to investigate whether various honey concentrations and larval ages affected the sex ratio of Betta fish. The result shows that both factors significantly affected male sex formation. The highest number of males was produced at a honey concentration of 40 mL/L. A significant difference in the number of males was shown in the larval groups of 3 and 4 days old, but not in the groups of 5 days old. The current research may contribute to the masculinization strategy in Betta fish to increase the number of male fish.

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