Masculinization of beta fish larvae *Betta splendens* through the different treatment immersion of honey solution and larval age

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**Abstract.** Betta fish *Betta splendens* have morphological differences between males and females. Male betta fish have more attractive body colors and shapes than females. This causes the selling value and market demand for males to be higher than that of females. Therefore, it is necessary to make efforts to get enough male fish through masculinization. So far masculinization or the sex reversal into males using androgen hormones before sex differentiation occurs. However, the use of steroid hormones has been banned and reduced, because they cause environmental and public health problems and are carcinogenic. This study used natural honey solution to determine the effect of length immersion time in the honey solution and to obtain a suitable larval age for masculinization on the early gonadal formation and larval survival after treatment. Masculinization was carried out using larvae in three treatments immersion time as follows 10 hours, 11 hours, 12 hours, and control. After that, the treatment was continued with the best results of the treatment using 7-day old larvae (D7), D9, and D11. Each treatment using 30 larvae was immersed in a honey solution of 3 liters with a concentration of 5 ml / L. The treatments were kept until 60 days before sex observation. Early observations of gonad formation were carried out after the larvae were D20 with HE-stained histology. The data obtained were analyzed using descriptive statistics and a chi-square test. The results showed that the treatment for 12 hours of immersion had the highest male sex ratio (93%). While the treatment on D7 larvae age in honey solution had the highest percentage in males (96%). It can be concluded that the increase in the percentage of male betta fish is influenced by the length of time immersion in the honey solution and the age of the larvae in the first week after hatching. All treatments did not affect the survival of betta fish larvae.

1. **Introduction**

Betta fish *Betta splendens* is one of the freshwater ornamental fish commodities in Indonesia and has high economic value. Betta fish consist of 3 groups, namely fighting bettas, ornamental bettas, and wild bettas. A decorative hickey is a type of hickey whose beauty lies in the shape of its tail when it expands. Currently, getting male fish naturally often has problems. Therefore it is necessary to make efforts to get enough male fish so that it is economically profitable. One of the efforts to obtain a male monosexual population in betta fish can be done by using the genital direction method. The genital direction technique is a technique to mass-produce single-sex organisms into only females or males. This technique is divided into feminization and masculinization. Feminization is the directing of an organism's sex cells from male to female, on the other hand, masculinization is the direction of an organism's sex cells from male to female.

Hormones that play a role in sex direction are steroid hormones. This hormone is used to stimulate sex hormones in male and female organisms [1]. Currently, the use of synthetic steroid hormones such as 17α-methyltestosterone [2, 3], 17α-ethyltestosterone, 17β-estradiol, diethylstilbestrol, and trenbolone acetate to produce monosexual populations has been prohibited and reduced, because it causes...
environmental and public health problems and is carcinogenic. It is known that a natural ingredient that has 
the same role as synthetic steroid hormones for masculinization is honey.

The market demand for male betta fish is increasing with the increase in the community's desire to 
maintain and beautify the room with aquatic biota. The effort to increase the male population is to mass-
produce male betta fish. The method that can be applied to the production of male betta fish is by directing 
sex cells or sex differentiation. Hence the increased market demand for male betta fish and the application 
of sex cell directing efforts to produce monosexual male populations will become a problem if this is not 
properly understood.

The existence of the effect of the length immersion honey solution on the masculinization process of 
betta fish B. splendens through several studies that have been carried out [4, 5, 6, 7, 8]. Likewise, the 
application time of these materials to test animals (fish) also varies. [8] used honey immersion time for 
goldfish of 2 hours, 6 hours, and 10 hours, while [7] used honey immersion time of 5 hours, 10 hours, and 
15 hours for betta fish. Meanwhile, several studies relating to different larval age variations in the 
masculinization process of each organism have been reported. The one that is done by [9] used 5 days, 10 
days, and 20 days of age on African catfish larvae. Based on this, research by applying the length of 
immersion time at different larval ages to betta fish needs to be known to obtain an effective masculinization 
process.

2. Materials and Method

2.1. Research time and site location
This research activity was carried out from February until August 2019. The research took place at the 
freshwater aquaculture unit of the Aquaculture Study Program, Fisheries and Marine Science Faculty, 
Pattimura University.

2.2. Materials and equipment
Materials and equipment used in the study are as follows: Plastic bags for packaging and bringing larvae to 
the research site, aquarium as a place for acclimatization of larvae, a plastic jar as a container for treating 
and rearing larvae, spoon to take and move larvae and mix honey, measuring glass jar to measure the volume 
of water, syringe to measure the dose of honey, thermometer to measure water temperature, a stopwatch to 
measure the length of immersion time, sample bottles to store samples. Microscope, object glass and cover 
glass as a place for observing larvae, camera for documentation, and stationery to record data. The following 
are the materials used in this research such as clean fresh water for rearing the larvae, betta fish larvae as 
experimental object, floral forest honey as a soaking material for masculinized larvae, egg yolk, artemia, as 
feed for the larvae, alcohol for preserving the larvae, cotton as a curing medium, detergent and chlorine for 
cleaning larval rearing containers.

2.3. Research design
This research was conducted initially using fertilized eggs. Betta fish hatching takes 1-2 days after 
fertilization. Larvae can swim after 2–3 days of hatching. For the next 3 days, the larvae are not given food 
because there are still energy reserves contained in the yolk sack that has been carried since hatching is still 
available. After the larvae are 3 days old (D3), the larvae are fed with egg yolk solution until the larvae are 
7 days old (D7). Artemia is given after the larvae are 8 days to 60 days old or during the rearing period. In 
this experiment, the sexes observed were male and female in four categories after being treated with 
immersion using a honey solution. The study was divided into two parts, consisting of immersion in a honey 
solution on betta fish larvae for different lengths of time. Application of the masculinization was carried out 
as follows: 10 hours, 11 hours, 12 hours, and control. After the treatment to produce the best percentage of
males, then continued with immersion treatment in a honey solution was carried out on larvae aged 7 days, 9 days, and 11 days. All treatments using three replicates. Many 30 larvae were immersed in a honey solution of 3 liters with a concentration of 5 ml / L. After the treatment is complete, the larvae are transferred to a rearing container until they are 60 days old (D 60) to observe the development of sex phenotypically or the appearance of secondary sex characteristics. Larvae are fed with a frequency of feeding 2 times/day ad libitum. Early observations of gonad formation were carried out after the larvae were 20 (D20).

2.4. Measuring parameters
The measured parameters were the sex ratio, survival rate, and early gonadal formation. Identification of sex in betta fish is carried out by morphological observations and secondary sex characteristics when the fish larvae are 60 days old. According to [10], the male sex ratio can be calculated using the following formulation:

\[
Sex \ ratio \ of \ male \ (%) = \frac{Number \ of \ male}{in \ total \ larvae \ sample} \times 100
\]

Survival is the percentage of the ratio of the number of initial larvae stocked in the treatment and the number of larvae at the end of rearing.

\[
SR \ (%) = \frac{N_t}{N_0} \times 100
\]

Explanation: SR = survival rate (%), No = the number of larvae at the beginning of the study, Nt = the number of larvae at the end of the study.

The initial determination of gonad formation for betta fish larvae was using the histological method on gonadal tissue at 20 days age. The method using gonad histological preparations with the Hematoxylin-Eosin staining.

2.5. Data analysis
The data obtained from the results of this experiment are categorized as nominal in the form of the frequency of possible appearance of male or female sex after being treated with a length time with a honey solution. Therefore, the data analysis used chi-square, \( \chi^2 \). The data were analyzed using descriptive statistics and displayed with tables and figures.

3. Result and Discussion

3.1. Percentage of male sex ratio of Betta fish larvae (B. splendens) with the treatment of different immersion time of honey solution and larvae age
The male sex ratio is the number of male fish compared to the total number of experimental fish. The results of sex ratio observations based on the census of secondary sex characteristics gave relatively good results. This is relatively easy to do when compared to the identification of primary sex traits. For the introduction of primary sex characteristics, it is necessary to carry out the process of observing the gonadal tissue through histological techniques. However, observations with this technique have several obstacles, namely that fish samples must be sacrificed for observation while the number of larvae is only small and the cost is not cheap. Sex identification can be done by looking at the morphology of secondary sex characteristics without killing the fish [11, 12, 13]. Secondary sex characteristics that are visible and can be observed are body shape, face shape, belly shape, tail fin shape, and white point of the genital tract.
The percentage of sex obtained in this study varied as shown in Table 1. Male sex variations obtained from the treatment ranged from 79% -97%. Except those without treatment (control) had the lowest male sex value (40%). This is also stated by [14], that in one spawning period, male betta fish usually reach 40% and 60% female. The results of the percentage of sex ratio shown in Table 1 show that the treatment of the duration of immersion of Betta fish larvae in honey solution for 12 hours has the highest male sex ratio value, reaching 97%. This is consistent with the chi-square analysis which shows a very strong dependence on the length of immersion time. According to [15], the best immersion time for masculinization of 5-day-old betta fish larvae is 10 hours with a male percentage of 92.13%. This was also revealed by [8] the best immersion time for masculinization of carp larvae is 10 hours with male sex reaching 100%. Meanwhile, the results of research by [6] found that the best honey dose was 5 ml / l with a long soaking time of 12 hours resulting in a male percentage of 77.33%.

### Table 1. Percentage of sex ratio betta fish larvae aged 60 days

| Treatment | Length of immersion (hour) | Larval age (day) |
|-----------|---------------------------|-----------------|
|           | Control                   | 10 H | 11 H | 12 H | Control | D7 | D9 | D11 |
| Male (%)  | 40                        | 79   | 88   | 97   | 97      | 34 | 96 | 90 | 83 |
| Female (%)| 60                        | 21   | 12   | 3    | 66      | 4  | 10 | 17 |

It is suspected that the body wall of the larvae is permeable so that it takes the right time for the honey solution to easily enter the larva's body [16]. The method of soaking larvae with honey solution can work effectively for the process of male sex differentiation by looking at the relationship between the concentration of the honey solution and the time of immersion [17]. The results of this study indicate that the longer of soaking time for the larvae using the honey solution, the higher the percentage of male larvae obtained. The percentage of male sex obtained from previous studies may differ from this study because of influenced by the following factors are differences in the type of fish used, the type of honey, and the age of the larvae.

Because the ratio of the male sex to the length of time soaking the honey solution for 12 hours produced the highest value (97%), further observations were made on the age of the betta fish larvae, as follows 7, 9, and 11 days. The results showed that the percentage of male sex ratios ranged from 83-96%. The highest percentage of male sex ratio was obtained in larvae aged 7 days (96%), followed by larvae aged 9 days (90%), and age 11 days (83%) (Table 1).

The factor causing the high percentage of male sex ratio is thought because the immersion in the honey solution was given at the right time at the age of the larvae of 7 days, 9 days, and 11 days when the larvae were in an unstable period. This period occurs in the process of determining and differentiating sexes. In this condition, the gonad genotype has been determined, but the development has not occurred. So that the treatment given can affect the development of gonads towards males.

The high percentage of male sex ratio obtained by immersing the honey solution for 12 hours in the 7-day betta fish larvae. This is thought because the body wall of the betta fish larvae is thinner than the 11-day larvae which have the lowest male sex ratio. [9] states that the older age of larvae, will have a lower percentage of males obtained. The effect of soaking honey on male differentiation is thought to occur when the honey enters the body and promotes blood circulation until it reaches the target organs. This happens because the body wall of the larvae is still permeable so that the water containing honey diffuses into the larva's body. In addition, [18] also states that the honey solution enters through the gills and lateral line. The
potassium content in honey that enters the larva's body can convert fat into pregnenolone is a source of biosynthetic steroid hormones by the adrenal glands. This steroid affects the formation of testosterone, so that fish that are initially female will be directed to become male [19]. Honey, which is used as a masculinization material, contains supporting compounds for the process of sex differentiation and naturally occurring in the environment. The chrysin content in honey is an inhibitor of the aromatase enzyme which plays a role in reducing the concentration of estrogen [10]. This results in the hormone testosterone which is a male hormone becoming active will influence and stimulate the development of male genitalia, secondary sex characteristics, and spermatogenesis [19].

3.2. The survival rate of betta fish larvae

The survival percentage of betta fish larvae after 10, 11, and 12 hours of honey immersion treatment as well as at 7, 9, and 11 days of age larvae and without treatment (control) has a value of 100%. While the percentage of survival in both treatments at the end of larval rearing for 60 days has a value ranging from 29% to 32% (Table 2).

| Percentage of larval survival | Treatment |
|------------------------------|-----------|
|                              | Length of immersion (hour/H) | Larval age (day/D) |
| At the start of treatment (%) | Cont. 10 H 11 H 12 H Cont.  D7 D9 D11 |
| After treatment (%)           | 100 100 100 100 100 100 100 100 |
| End of larval rearing (%)     | 33 32 29 32 30 31 30 30 |

The results of previous studies indicated that the survival rate of betta fish larvae with the honey solution immersion method varied, among others, 79.17% -100% [13]; 53.33% -83.33% [14]. According to [20], the percentage of survival of betta fish larvae that are reared until the age of 6 days ranging from 28.38% to 59.90%. This is presumably because the concentration and content of masculinizing materials used can cause stress and death in fish larvae. When compared with the results of this study, the percentage of survival at the beginning and after immersion treatment in honey solution also different larval ages had a high value of 100% (Table 2). It is thought that the immersion time as well as the age of the betta fish larvae used, can still be tolerated so do not mortality occurred.

During the 60 days rearing period, the survival of betta fish larvae tends to low. This is due to internal factors and external factors. The internal factor comes from the fish larvae themselves, such as the physical condition of the larvae and the ability to use their food. Meanwhile, the external factors that must be considered during the rearing of betta fish larvae are take of feeding according to the larva's mouth sizing, the availability of feed in the rearing media, the available enough living space, handling of larvae and water quality. Besides, it is presumed that betta fish larvae experience high mortality due to the formation of the labyrinth as an additional breathing apparatus in the second until the third week. Anatomically it is a complex bony cavity structure. This additional breathing apparatus is used to take oxygen directly from the air. During the labyrinth formation process, betta fish larvae are in a critical phase so they are susceptible to environmental changes and are easily stressed. This is according to [6], resulted in a low survival rate of fish larvae. However, based on the results of the chi-square analysis, it is known that there is no effect or dependence on the long soaking time of honey on the survival of betta fish larvae during the rearing time.
3.3. Early gonad formation of larval betta fish
Observation of betta fish gonads was carried out when the larvae were 20-days old. Observation of betta fish larvae in this study was to show the gonad tissue of the newly formed larvae through histology. Based on the results of observations using a microscope with a magnification of 40 times, it was seen that there were differences in male and female gonads (Figure 1).

![Characteristics spermatocyte in male gonad](Image1)
![Characteristics oocyte in female gonad](Image2)

**Figure 1.** Histology gonads of *Betta splendens* with Hematoxylin-Eosin Staining (magnification of 40 X).

In Figure 1, the female gonad shows a spherical ovule with the cell nucleus in the center and surrounded by cytoplasm. Male gonads were found in the presence of spermatozoa which appeared much smaller than the egg cells and had a large number and looked like evenly spread spots. The process of forming female gonads is thought to begin with meiosis oogonia or multiplication of somatic cells in the form of ovary cavities [15]. Meanwhile, the process of male gonad formation is characterized by the appearance of spermatogonia. This shows that the masculinization of betta fish larvae with a honey solution was successful when the gonads were not yet differentiated.

4. Conclusion
Based on the results of this study, the increase in the percentage of male sex ratio of *Betta splendens* was influenced by the length of immersion time in the honey solution and the age of the larvae but did not affect the survival rate of the larvae. Soaking the honey solution in betta fish larvae aged 7-days for 12 hours had the highest male sex ratio values (93% and 96%), respectively. Meanwhile, the formation of gonads for betta fish begins when the larvae are 20 days old. It can be concluded that masculinization through soaking honey solution in betta fish larvae was successfully carried out in the first week after hatching.

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