# ANALYSIS OF MILT QUALITY AND TESTIS HISTOLOGICAL FEATURE OF POST-SPAWNING BAWAL (*Colossoma Macropomum*)

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**Abstract.** The research aims to observe several reproductive parameters of Bawal/pomfret fish after spawning. Data on milt quality parameters were crucial for determining the readiness of male parent for successful spawning, while ensuring female parent has reached sexual maturity. The observation results on the quality of post-spawning male Bawal from week 0 (M0) to week 3 (M3) are as follows, milt volume=0.1 - 1.75 ml, Milt pH=6 - 7, sperm motility=35 - 100%, and the density or number of spermatozoa ranged from 2.03 x $10^{11}$ cells/ml (M0) to 3.56 x $10^{11}$ cells/ml (M2). Furthermore, the length of spermatozoa ranged from 40 to 60 µ, with a head length of only 2-3 µ. Supporting data on GMI of male parents was 2.57% (M0) - 4.02% (M2). The histological observations of testicular development showed that all stages of spermatogenesis were visible from M0 to M3 preparations, including spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. The proportions of each stage of spermatogenesis are as follows, (in %): M0 (spg=13.5; sp=15.3; ss=17.8; smt=15.1; spz=63.2); M1 (spg=5.3; sp=4.2; ss=10.6; smt=14.4; spz=61.2); M2 (spg=5.2; sp=6.3; ss=9.9; smt=9; spz=55.1); M3 (spg=6.0; sp=5.0; ss=30.5; smt=17.1; spz=52.5). Despite the good quality status of milt and GMI, only 4 out of 24 post-spawning male Bawal produced milt during stripping (M0-1, M0-2, M2-4, and M3-4), while the other 20 did not. This correlated with data obtained from Bawal farmers in Banyumas, Central Java, who reported that during the dry season, male Bawal had difficulty preparing for spawning, even when their reproductive quality status was relatively good.

**Keywords:** histology, milt, motility, spermatogenesis, striping, testis

## A. Introduction

Successful fisheries cultivation depends on the availability of adequate and sustainable seeds. The development requires a consistent supply of good quality seeds in large quantities, regardless of the season, which usually requires successful mating or spawning. In seed production activities, it is important to have male and female parents who are reproductively qualified, with mature gonads to ensure readiness for mating/spawning and produce high-quality seeds in sufficient quantities.

This study was inspired by insights from the Management of Mina Rahayu Fish Farmers Partners in Purwosari Village, Purwokerto Utara District, Banyumas Regency (oral discussion, mid-October 2022), specifically the cultivation of freshwater Bawal (*Colossoma Macropomum*). A key issue identified was male fish struggled to produce sperm/milt or mate/spawn during the dry season, leading to a delay in gametogenesis. The unpreparedness among male fish disrupts production during this season. In this context, the study aimed to investigate the basic profile of the quality of milt in male Bawal and test the preservation

techniques to ensure sperm/milt can be stored and used over an extended period, making the spawning process less dependent on seasonal variations.

Information on the reproduction of freshwater Bawal in scientific journals published in Indonesia is still limited. Most publications focus on aspects such as population parameters, seeding, enlargement and growth, as well as post-harvest, while foreign publications discuss more about sea Bawal. Moreover, there is little to no published study on cases where male Bawal experiences delayed sexual maturation or insufficient milt production, with similar issues reported by related journal references. This makes the problem interesting to analyze further.

In the aspect of reproduction, there is a possibility that the gonad maturation period varies between male and female Bawal. This lack of synchronization, as reported by farmers, can significantly disrupt the production cycle, since male freshwater Bawal rarely releases milt during the dry season when stripped. Therefore, this study aimed to intensively investigate the lack of synchronization challenge.

The quality of milt, as gametes from male fish, is a crucial factor in preparing for spawning with female fish, alongside the readiness factor of female's gonad maturity. Milt quality is typically assessed based on observable characteristics, particularly motility. This can be determined by measuring the percentage of motile milt and the duration of its motility, which refers to the time each progressive movement lasts until it stops.

## B. Methods

### 1. Time and Place of Study

A survey was conducted at the Animal Structure and Development Laboratory, and the Experimental Station for the Diploma (D3) Fish Cultivation Program, Faculty of Biology, Jenderal Soedirman University (Unsoed), as well as in several natural ponds in Sumampir Village, Banyumas, for 6 months, starting from May 2023 to November 2023. Male parents samples were obtained from partner spawning ponds at Singosari Village, Banyumas Regency, and acclimatized for 5 days at the Experimental Station of the D3 Fish Cultivation Study Program, Faculty of Biology, Jenderal Soedirman University. Spawning and maintenance of post-spawning fish samples were carried out at the Experimental Station of the D3 Fish Cultivation Study Program, Faculty of Biology, Jenderal Soedirman University. Furthermore, evaluation of milt quality, histology preparation, and data analysis were carried out at the Animal Structure and Development Laboratory, Faculty of Biology, Jenderal Soedirman University. This study was conducted for 6 months, namely from June 2023 to November 2023.

### 2. Materials and Tools

The materials used in this study were male freshwater Bawal with mature gonads, aged 20 months and not previously spawned, as well as female freshwater Bawal with mature gonads, aged 36 months and previously spawned several times to stimulate the spermiation process. Female freshwater Bawal had an average weight of 4.35 ± 0.29 kg, while males weighed 3.45 ± 0.32 kg, with gonad maturity of GMI IV. Other materials included handscoon, gonadotropin-releasing hormone (GnRH) analog + domperidone (Ovaprim Syndel Canada), 10% Neutral Buffered Formalin (NBF) solution, graded alcohol (70%, 80%, 90%, and absolute), paraffin (melting point 56°C), xylol, aquades, new entellan, Harris hematoxylin, 1% eosin, gelatin powder, universal pH indicator paper, 0.9% NaCl solution, spirits, LP 3-5 fish food pellets, iodized salt, tissue paper, and label paper.

The tools used included a permanent pond measuring 10 x 4 x 1 $m^3$ divided into 10 chambers measuring 2 x 2 m with bamboo partitions, 3 units of aquariums/fiber tubs measuring 230 x 120 x 60 $cm^3$ equipped with an aeration system, aquarium cover nets, cloths, surgical instruments, 50 ml sample bottles, digital scales with an accuracy of 0.01 g, 1 kg sitting scales,

1 ml and 3 ml injection syringes (without needles), plastic cups, hemocytometers, wellplates, object glasses, cover glasses, beaker glasses, cavity slides, tissue cassettes, embedding molds, paraffin dispensers, staining jars, preparation trays, light microscopes, eyepiece graticules, rotary microtomes, incubator ovens, basins for waterbath modifications, hand-counters, aluminum foil, bunsen burners, large cutters with a blade width of 18 mm and a blade thickness of 0.5 mm, Optilab cameras, container boxes, as well as stationery.

3. Experiment Design

This study was conducted using a survey (observation) method. The samples were 24 mature males and 4 females freshwater Bawal used to stimulate milt process. After spawning, male samples were separated from females and eggs. Subsequently, relevant data were obtained from male fish in weeks 0, 1, 2, and 3. A total of 24 male fish samples were divided into 4 groups based on the time of data collection, with each group comprising 6 male fish randomly taken for evaluation of milt quality and 3 used for histological preparations. The sample grouping is as follows:

a. $M_0$: group of male freshwater Bawal on 0 weeks after spawning.
b. $M_1$: group of male freshwater Bawal on 1 week after spawning.
c. $M_2$: group of male freshwater Bawal on 2 weeks after spawning.
d. $M_3$: group of male freshwater Bawal on 3 weeks after spawning.

4. Variables and Parameters

The variables used were milt quality and the development of spermatogenic cells of freshwater Bawal during 0, 1, 2, and 3 weeks after spawning. Furthermore, the parameters observed included milt volume, pH, color, density per ml, percentage motility, general description of the testis structure, and the proportion of lobules at each spermatogenic stage.

5. Implementation

a. Preparation of Freshwater Bawal After Spawning

Mature male and female freshwater Bawal were obtained from the spawning pond of Singosari Village, Banyumas District, and acclimatized for 5 days in a 230 x 150 x 60 $cm^3$ fiber tub. This location was used for parents spawning at the Experimental Station of the D3 Fish Cultivation Study Program, Faculty of Biology, Jenderal Soedirman University.

Spawning method used was semi-natural. This was implemented by injecting female and male parents with the hormone ovaprim syndel to stimulate ovulation and spermiation in parents whose gonads have previously matured, subsequently left to spawn naturally in a spawning container [30].

Each fiber tank unit was filled with 8 male and 2 female parents. In addition, fish were induced with Ovaprim to stimulate gamete maturation and milt production [10]. Ovaprim is the trade name for GnRH analog hormone containing 20 µg salmon GnRH (sGnRH) and 10 µg domperidone. According to [11], sGnRH hormone in Ovaprim is an analog of GnRH hormone that directly stimulates the pituitary gland to secrete luteinizing hormone (LH), accelerate spermiation, and increase milt production. LH indirectly played a role in the process of spermiation and hydration of milt, producing domperidone content (anti-dopamine) which functioned to block dopamine and inhibit LH secretion. The total dose of Ovaprim to be used for induction was 0.5 ml/kg of fish body weight. Ovaprim was also diluted using 0.9% NaCl solution with a volume ratio of 1:1.

The dose of hormone injection was 0.35 ml/kg, performed at a 45° angle in the back muscle of parents. Female parents were injected twice at an interval of 6 hours, with the first injection at a dose of 0.50 ml/kg and the second at 0.35 ml/kg. Male parents received a single injection

at the same time as female's first injection, with a dose of 0.50 ml/kg [31]. Furthermore, female fish were injected twice with an interval of 8 to 12 hours, where the first injection was administered at 2.00 p.m., and the second at 8.00 p.m.

Male and female freshwater Bawal were injected with Ovaprim 10-12 hours before spawning. The injection procedure was carried out intramuscularly about 0.5-1.0 cm below the dorsal fin, with a slope of 30°-45° toward the head [13]. Furthermore, the induced fish were released into two fiber aquariums, each measuring 230 x 120 x 60 cm with a density of 12 male and 2 female fish per fiber tank. After spawning, samples of male fish were separated from female fish and eggs and immediately transferred to a permanent outdoor pond.

b. Freshwater Bawal Maintenance After Spawning

After spawning, male freshwater Bawal broodstock was moved from the fiber spawning tank to a permanent outdoor pond (10 chambers) measuring 10 x 2 x 1 $m^3$. Each chamber, measuring 2 x 2 x 1 $m^3$, was filled with freshwater Bawal broodstock with a density of 2 fish and covered with a net. During maintenance, fish were fed with LP 3-5 pellets of approximately 5% of the biomass weight of fish. Feeding was carried out at 07.00 and 17.00 WIB. Fish should be kept in healthy condition and not physically disabled while waiting for each post-spawning period.

c. Milt Quality Evaluation

Fresh quality was evaluated to determine the characteristics of freshwater Bawal milt macroscopically and microscopically. Macroscopic evaluation was carried out by measuring milt volume, pH, and color, while microscopic evaluation included measuring the density per ml and percentage of motility [38]. Sampling was carefully conducted manually by sorting (stripping). This commenced by cleaning the abdomen and urogenital pores using tissue paper to prevent contamination of urine, feces, blood, or water [37], and slowly massaging the abdominal wall of fish toward the urogenital pores until white fluid (milt) comes out and is sucked using a needle-free syringe.

1) Milt Volume Measurement

Freshwater Bawal milt volume was measured using a 3 ml injection syringe without a needle. Milt that came out of stripping results was sucked using a syringe. The volume was measured based on the scale listed on the syringe and subsequently collected with a plastic cup.

2) Milt pH Measurement

The pH value of milt was measured using universal indicator pH paper. One strip of pH paper was dipped into milt and a color change was observed. The strip was subsequently read by comparing to the indicator scale table 1-14 listed on pH paper container.

3) Observation of Milt Color

The color was observed by directly examining milt collected in a plastic cup. According to [7], the color of fresh milt in good quality freshwater Bawal is milky white.

4) Milt Density Calculation

Density was calculated using a hemocytometer. A total of 0.1 ml was collected using a 1 ml injection syringe without a needle, and diluted in a wellplate. The ratio of the volume and 0.9% NaCl diluent solution was 1:9. The same ratio was applied for subsequent dilutions up to 10,000 times. Subsequently, the diluted milt was dripped into the hemocytometer counting chamber covered with a glass. Generally, the hemocytometer comprised nine large boxes. Milt cells were counted using the middle box divided into 25 boxes. The counting was carried out diagonally in 5 boxes (Figure 1), namely 4 boxes in the corners and 1 box in the middle. Moreover, the average number of cells from the 5 boxes was determined [12].

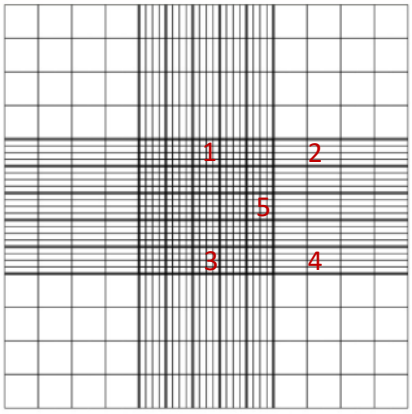


Figure 1. Hemocytometer Counting Room. Source: [39].

Observations were made under a microscope with a magnification of 400x. Furthermore, the density of milt was calculated using the following formula:

$$\text{Cell density} = n \times 0{,}25 \times 10^6 \times p \quad (1)$$

**Description**:

n: Average number of spermatozoa cells

p: Number of dilutions

5) Measurement of Milt Motility Percentage

Motility percentage was measured by observing the movement of milt in the first 15 seconds after being activated by adding water to the cavity slide. A drop of 100-fold dilution milt was activated using water, and subsequently stirred gently and evenly. The volume ratio of the diluted milt and water was 1:1. Motility observation was carried out under a microscope equipped with a graticule eyepiece. Motility observation process should be carried out immediately. The quick process could be attributed to the very short movement of milt cells, specifically in freshwater fish which only survive for about 40-60 seconds after being activated with water [13]. The percentage of milt motility was calculated using the following formula:

$$\% \text{ motility} = \frac{\sum kotak\ berisi\ spermatozoa\ motil}{\sum total\ kotak\ berisi\ spermatozoa} \times 100\% \quad (2)$$

Milt motility value was calculated based on the following criteria:

Table 1. Criteria for spermatozoa movement level

| Criteria | Score |
|---|---|
| When observed > 70% of milt cells move. | 4 |
| When observed 50-70% of milt cells move. | 3 |
| When observed 25-50% of milt cells move. | 2 |
| When observed only 1-25% of milt cells move. | 1 |
| 0% or all milt cells do not move. | 0 |

Source: [40].

6) Gonad Maturity Index (GMI)-Supporting Data

GMI is a comparison between the weight of the gonads to the body of fish. The increase in GMI in line with the maturity level of fish gonads was determined using the following formula [4]:

$$GMI = \frac{BT}{BG} \times 100 \quad (3)$$

Description:
GMI : gonad maturity index (%)
BG : fish gonad weight (g)
BT : fish body weight (g)

d. Observation of Testis Histological Structure

e. Preparation of Testis Histological Preparations

Male parents were dissected, and testis was taken for histological preparations, performed using paraffin method (embedding). Testis samples were obtained by dissecting the body of fish in the urogenital porus section at the anterior and the medio-ventral directions.

The partitioned male gonads (testis) were kept in sample bottle containing 10% NBF fixative solution, subsequently processed using the paraffin method described by Suntoro (1987), with modifications by the Structure and Development Laboratory of the Faculty of Biology, Jenderal Soedirman University [14].

f. Testis Histological Observation

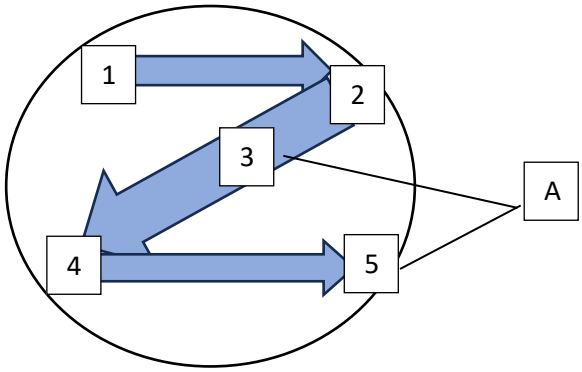


Figure 2. Field Observation of Testis Histological Preparations.

Description: (A) Field of observation on a microscope; (1, 2, 3, 4 & 5) Field of view

Testis histological preparations of freshwater Bawal were observed under a light microscope. Each section of male gonad preparation (anterior, medial, and posterior) was observed in 5 slices, and for each slice, 5 microscopic fields of view were observed with a Z pattern. The method for determining the field of view is presented in Figure 2.

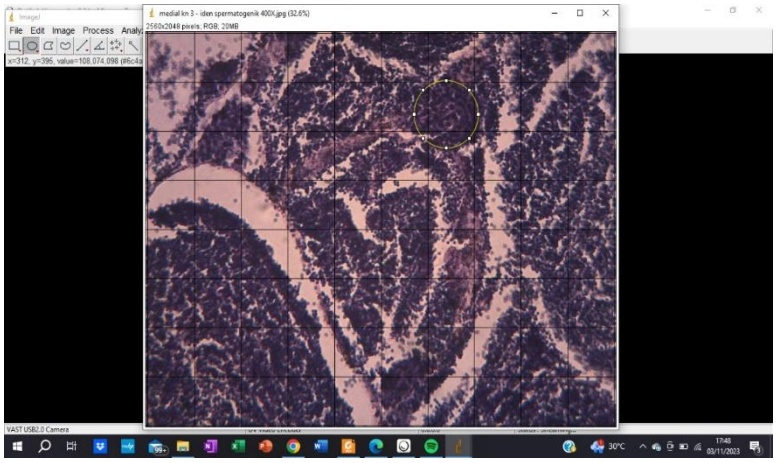

Figure 3. Display of the method for calculating the proportion of each spermatogenesis stage using OptiLab with the help of Image-J software and the grid feature.

Testis histological preparations of freshwater Bawal were observed under a light microscope at 400x magnification using an Optilab camera. Each section of male gonad preparation (anterior, medial, posterior) was observed in 5 slices, and for each slice, 5 microscopic fields of view were observed with a Z pattern. Identification of the proportion of lobules at each spermatogenic stage in each field of view was assisted by using Image-J software with a grid feature. The proportion of the lobula area occupied by each particular spermatogenic stage was calculated using the 3-3 formula. Image-J software and the Grid feature helped ensured that the fields of view counted previously were not recounted (Figure 3).

Identification of the lobules proportion at each spermatogenic stage included spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. According to [9], milt cells in gonads of *Cyprinidae* fish were developed in 5 stages, each with different characteristics, spermatogonia were located near the basal membrane, were light-colored granules with nucleoli, and were basophilic with pale nuclei; primary spermatocytes were large cells with large, round, basophilic nuclei; secondary spermatocytes were about half the size of primary spermatocytes; spermatids had oval-shaped cells; and spermatozoa had flagella. The proportion of the lobula area occupied by each particular spermatogenic stage was calculated using the formula from [15], as follows:

$$X = \frac{\sum area\ lobula\ dengan\ tahapan\ sel\ spermatogenik\ tertentu}{\sum area\ lobula\ yang\ diamati} \qquad (4)$$

**Description:**

X : Proportion of lobula area of each specific spermatogenic stage

6. Data analysis

The data used in this study were qualitative and quantitative. The qualitative data included visual observation of milt color and histological description of testis analyzed descriptively and presented in the form of photomicrographs. Meanwhile, quantitative data included milt volume, density, and the proportion of each spermatogenic stage, milt pH, percentage of milt motility and supporting GMI data presented in the form of bar charts, analzed subjectively. Qualitative data from the identification of histological features of freshwater Bawal gonads were analyzed descriptively, referencing gonads [4] and the histological features of the testis (male gonads) in Teleost fish [5].

## C. Results And Discussion

The discussion of the three aspects of milt quality parameters, GMI value, and the development of testis histological features, specifically the proportion of each spermatogenesis stage, cannot be separated in this study, as these three aspects influence and are influenced by each other. Milt quality is a measure of its ability to successfully fertilize an egg. This ability is largely dependent on the qualitative parameters of milt, namely the composition of the seminal fluid, milt volume, density and motility [20].

Table 1. Summary of Data Analysis of Parents Male Freshwater Bawal Milt Quality

| SAMPLE CODE | | PARAMETER | | | | | SUPPORTING DATA | | Gonad Maturity Index |
|---|---|---|---|---|---|---|---|---|---|
| Fish | Spawning Week | Volume (ml) | pH | Color | Milt density cells/ml | % Motility | Fish weight (g) | Gonad weight (g) | |
| 1 | M0 | 1,75 | 7 | Milky white | 3.23 x $10^{11}$ | 100 | 3170 | 113 | 3.56 |
| 2 | M0 | 0,7 | 7 | White | 2.86 x $10^{11}$ | 90 | 3300 | 108 | 2.57 |
| 3 | M0 | - | - | - | 0 | 0 | 3350 | 105 | 3.13 |
| 4 | M0 | - | - | - | 0 | 0 | 3150 | 130 | 3.125 |
| 1 | M1 | - | - | - | 0 | 0 | 3460 | 86 | 2.48 |
| 2 | M1 | - | - | - | 0 | 0 | 3380 | 101 | 2.91 |
| 3 | M1 | - | - | - | 0 | 0 | 3400 | 110 | 2.61 |
| 4 | M1 | - | - | - | 0 | 0 | 3040 | 103 | 3.38 |
| 1 | M2 | - | - | - | 0 | 0 | 2780 | 131 | 4.00 |
| 2 | M2 | - | - | - | 0 | 0 | 2680 | 91 | 3.30 |
| 3 | M2 | - | - | - | 0 | 0 | 3230 | 130 | 4.02 |
| 4 | M2 | 0.7 | 7 | Milky white | 3.56 x $10^{11}$ | 65 | 2940 | 95 | 3.23 |
| 1 | M3 | - | - | - | 0 | 0 | 2870 | 110 | 3.83 |
| 2 | M3 | - | - | - | 0 | 0 | 3410 | 143 | 4.20 |
| 3 | M3 | - | - | - | - | 0 | 3420 | 94 | 2.74 |
| 4 | M3 | 0.1 | 7 | Clear yellowish | 2.03 x $10^{11}$ | 35 | 2870 | 109 | 3.79 |

FEMALE BAWAL PARENT DATA

| No | Code | Weight (g) | Gonad (g) | GMI |
|---|---|---|---|---|
| 1 | BB1 | 4430 | 238 | 5.57 |
| 2 | BB2 | 3230 | 198 | 6.13 |
| 3 | BB3 | 4005 | 285 | 7.11 |
| 4 | BB4 | 4030 | 305 | 7.56 |

**Description:**

$M_0$ : Spawning Week 0

$M_1$ : Spawning Week 1

$M_2$ : Spawning Week 2

$M_3$ : Spawning Week 3

Table 1 shows the volume of milt obtained at a range of 0.1-1.75 ml. The volume was measured using syringe to collect the milt that expelled. According to [16], fish milt is considered normal when the volume ejaculated by stripping is a maximum volume of 1 ml. This showed freshwater Bawal milt observed in this current study was generally in the normal range

during the post-spawning striping procedure. According to [17], the size of spermatozoa in Teleost fish ranges from 40-60 µm, with relatively high milt production.

Table 1 further shows that during the 0-week post-spawning period, 2 out of 4 male fish stripped to collect milt could not produce any milt. In the first week (M1), none of the 4 test fish samples succeeded in producing milt. In the second week (M2), only one test fish sample produced milt. Similarly, in the third week (M3) post-spawning period, only one test fish sample managed to produce milt. Figure 4 also shows milt volume of freshwater Bawal tested.

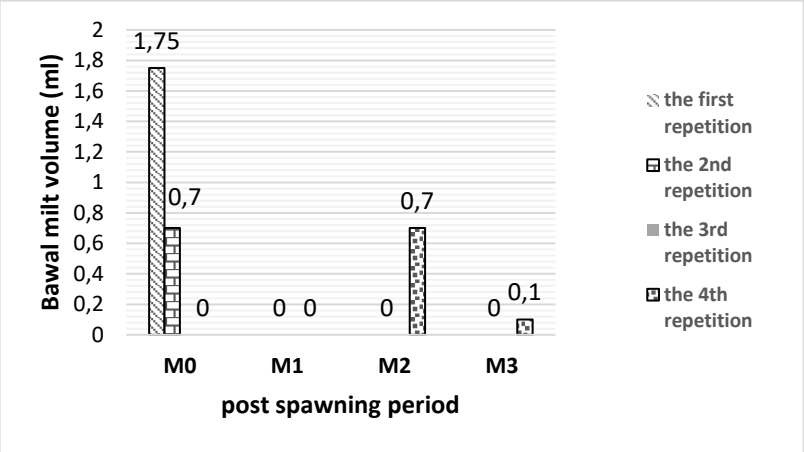


Figure 4. Freshwater Bawal milt volume (ml) in 4 post-spawning periods ($P_0$, $P_1$, $P_2$, $P_3$)

Based on pH parameters, freshwater Bawal milt had value between 6 - 7, meaning that the pH was less than normal. According to [18], milt tends to live longer at neutral pH. The normal pH condition, measured immediately after liquefaction, was weakly alkaline. However, the pH can change over a long period.

The observation results of freshwater Bawal milt based on microscopic parameters obtained 35-100% motile milt, and the rest were non-motile. Furthermore, the density or number of spermatozoa was 2.03 x $10^{11}$ cells/ml (group after spawning week 0) - 3.56 x $10^{11}$ cells/ml (group after spawning week 2), and the color was clear, clear yellowish, yellowish, white, and grayish white. According to [19], milt morphology, besides density of motile milt, is a parameter of milt analysis, a good indicator of quality. Moreover, [16] found that normal motility decreased 2-3 hours after ejaculation, with a motility rate of around 50%-60% being considered good. A decrease in motility below 50% after 3 hours could signify a disorder or abnormality in the genitalia.

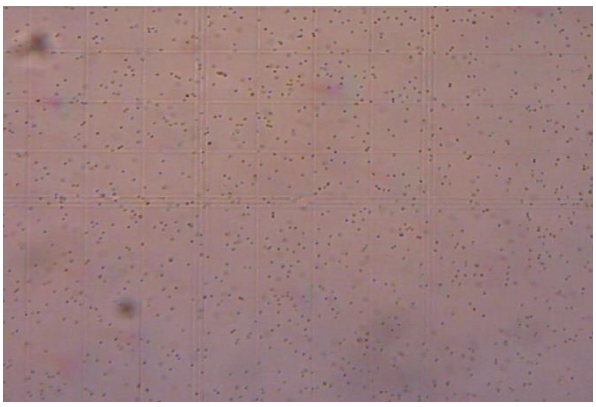

Figure 5. Appearance of freshwater Bawal milt in the M0_1 sample group

Fish milt is usually immotile and inactive in the testis. Motility begins after spermiation stage, when milt enters the aquatic environment of female reproductive system. At this stage, activity may occur when the pressure factor is diluted, pH becomes alkaline, and the osmolality becomes hypotonic. The average total length of Bawal milt was 40-60 µ with a head length of only 2-3 µ. Although the size and shape differ in fish species, the morphological structure is similar. Also, the surface of milt is wrapped by a lipoprotein membrane, and when the cell dies, the membrane permeability increases, specifically around the head base. The change in permeability is the basis for semen coloration that distinguishes between living and dead milt [20].

According to [21], milt quality in fish should be analyzed for artificial fertilization process and to determine the fertility of fish. This is because the role of milt as male gametes is important for the success of the emergence of new individuals.

According to [22], the life span of milt released from the testis is highly dependent on the energy contained in the spermatozoa body. The main material used as an energy source from outside testis is fructose, which is converted into lactic acid and energy with the help of the enzyme fructolysin in the glycolysis process. The decrease in the percentage of life in the storage process can also be caused by milt metabolism which produces by-products in the form of lactic acid or carbon dioxide ($CO_2$). In addition, acidity level can inhibit milt metabolic activity.

Sufficient quantity and good quality of milt are crucial factors for proper fertilization process of fish species. Several studies have been carried out to describe the characteristics of milt quality, including endurance, motility, and composition of the fluid. Moreover, motility is defined as the ability to actively swim in a forward motion. Milt fluid has unique organic and inorganic components [24]. According to [23], organic and inorganic components significantly affect the quality of milt. Examples of these components are minerals (potassium, sodium, magnesium, calcium and chloride), pH, protein, glucose and triglycerides. However, the composition of milt fluid, motility, and endurance significantly affect the ability to carry out the fertilization process.

Fish seminal fluid has a unique composition regarding the presence of organic and inorganic components that support the survival of milt [25]. Milt motility and density determine the fertilization ability of milt and are often used to estimate quality [6] due to the chemical properties of milt fluid. Therefore, fish milt is not motile in the seminal fluid. During natural spawning, fish milt become motile after being released into the aquatic environment (in oviparous species) or female genital tract (in viviparous and ovoviviparous species).

Hormonal spawning induction is an effective strategy to shorten and synchronize gamete maturation in the hatchery. In addition, hormone therapy can affect milt quality. The injection of European catfish (*Silurus glanis*) with carp pituitary extract (CPE) and implantation with GnRHa (gonadotropin-releasing hormone analog) were found to increase milt density. However, CPE had a greater effect [26].

Similar results were found when male carp were treated with oral and intraperitoneal administration of salmon analog (Ovaprim) gonadotropin-releasing hormone (sGnRHa) and Pimozide (Pim) [27]. In Caspian Brown Trout (*Salmo Trutta Caspius*), milt count, volume, motility, and pH increased with GnRHa induction [28]. Increased milt volume and prolonged spermiation were also observed in GnRHa (Ovaprim)-induced sea bass (Dicentrarchus labrax). GnRHa microspheres significantly increased milt production in Atlantic salmon (*S. salar*) and Striped bass (*Morone saksatilis*) [29].

1. Gonad Maturity Index (GMI)

Table 4.1 shows GMI status of each observed male freshwater Bawal after spawning. In terms of quality, the tested freshwater Bawal had a good GMI, with a mature gonad status. Table 4.2 shows the comparative GMI of black freshwater Bawal.

Table 2. Gonad Maturity Index of Black Freshwater Bawal [32].

| TKG | GMI | |
|---|---|---|
| | Male | Female |
| I | 0.06-0.14 | 0.08-0.09 |
| II | 0.05-0.68 | 0.34-0.90 |
| III | 1.03-2.89 | 0.72-2.04 |
| IV | 2.26-3.53 | 1.33-5.71 |

Source: [32]

GMI value of freshwater Bawal test in this analysis corresponded with GMI of Black Freshwater Bawal observed in [32], as shown in Table 2.

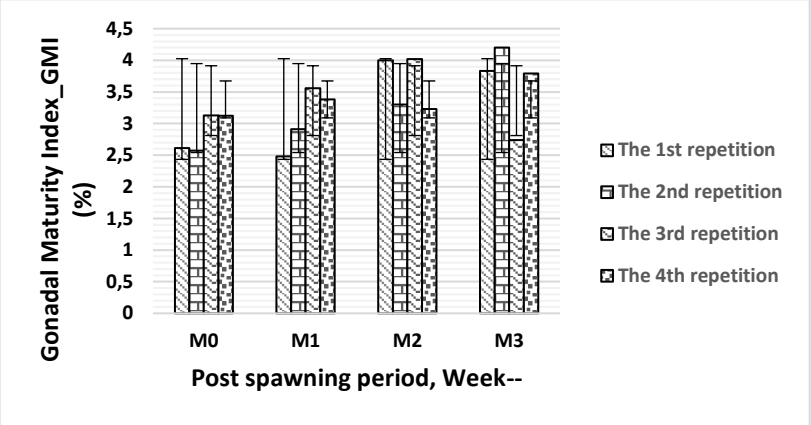


Figure 6. GMI Value of male freshwater Bawal tested in each post-spawning period.

2. Histological Description of Freshwater Bawal Parent Testis After Spawning

The data description (Table 1) and milt quality parameters of freshwater Bawal tested after spawning provided a general overview of the observations of post-spawning male Bawal during weeks 0, 1, 2, and 3. However, these results contrasted with the GMI value for each male fish tested (Table 4., Figure 4., and Figure 6).

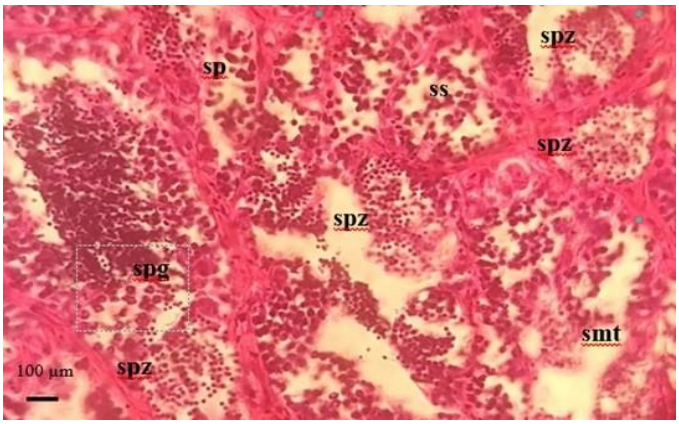


Figure 7. Microphotography of the testis of male freshwater Bawal after spawning, week 0. Repeat/sample 1

The microscopic observations of the testis preparations for each week of the post-spawning period supported the GMI value and strengthened the suspicion that post-spawning males were unable to ejaculate, whether naturally or through artificial spawning techniques like stripping.

This current study was conducted from July to October 2023, during the dry season in Banyumas area and its surroundings. Partner farmers had previously reported the inability of male freshwater Bawal to spawn during this season. From the start of parent selection, fish could not always produce milt, showing a lack of readiness to spawn. Micrographic photographs of testis histological preparations of male freshwater Bawal for each post-spawning period are as follows.

Figure 7 shows the histological structure of freshwater Bawal testis after spawning, all stages of spermatogenesis namely spermatogonium, primary spermatocytes, secondary spermatocytes, spermatin, and spermatozoa, were at their peak in the control group (week 0, before spawning). This correlated with GMI value of 3.56 shown in Table 1, signifying the relatively high GMI for male freshwater Bawal. For comparison, GMI for male black freshwater Bawal in [32] ranged from 2.26 to 3.53 at GMI IV, as shown in Table 2.

Based on Figure 8, the microphotography of male freshwater Bawal testis in the third week after spawning (sample 3), the histological description was similar to that of the control group in Figure 7. All stages of spermatogenesis are presented as follows.

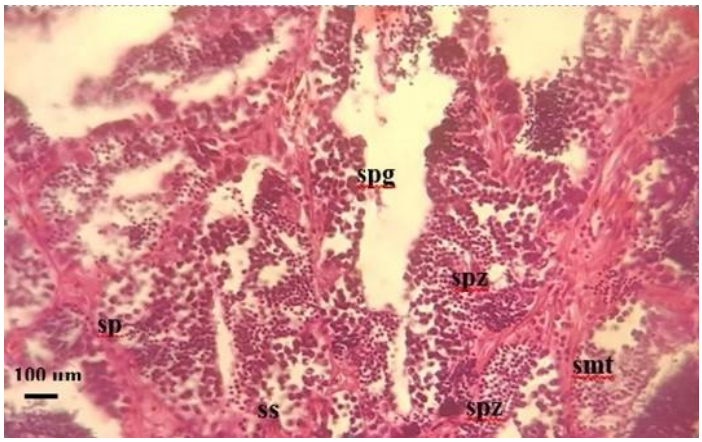


Figure 8. Microphotography of the testis of male freshwater Bawal after spawning, third week period, repeat sample 3

**Description:**

Spg = spermatogonium; sp = primary spermatocyte; ss = secondary spermatocyte; smt = spermatid; spz = spermatozoa (scale bar = 100 µm

The proportion of each spermatogenesis stage can be calculated through microscopic observation. The proportion of each stage of spermatogenesis in each post-spawning period of male freshwater Bawal is shown in Table 3.

Table 3. Proportion of Each Spermatogenesis Stage in Each Post-Spawning Period of Male Bawal

| Post spawning | Spermatogenesis stage | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | spg | | | | sp | | | | ss | | | | smt | | | | spz | | | |
| Repetition | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| M0 | 13.1 | 13.7 | 13.8 | 13.2 | 15.4 | 14.8 | 15.8 | 15.1 | 16.6 | 18.4 | 18.8 | 17.3 | 16.7 | 13.6 | 15.5 | 14.4 | 63.3 | 55.4 | 66.5 | 67.4 |
| M1 | 5.3 | 4.2 | 6.2 | 5.6 | 2.8 | 4.3 | 5.7 | 4.1 | 11.5 | 9.9 | 11.7 | 9.2 | 13.5 | 14.7 | 14.6 | 14.8 | 55.6 | 66.3 | 62.9 | 59.8 |
| M2 | 4.8 | 6.3 | 4.4 | 5.2 | 4.8 | 6.2 | 7.5 | 6.5 | 7.9 | 13.7 | 8.5 | 9.3 | 9.6 | 8.2 | 9.4 | 8.7 | 58.9 | 57.7 | 51.5 | 52.3 |
| M3 | 5.9 | 6.0 | 7.0 | 6.4 | 3.4 | 4.5 | 7.6 | 4.4 | 9.6 | 12.5 | 8.4 | 10.8 | 18.1 | 16.3 | 17.2 | 16.8 | 53.2 | 50.3 | 52.3 | 54.2 |

**Description:**

Spg = spermatogonium, sp = primary spermatocyte, ss = secondary spermatocyte, smt = spermatid, spz = spermatozoa

Spermatogenic cells in Teleost fish consist of spermatogonia, primary and secondary spermatocytes, spermatids, as well as spermatozoa [33]. Each spermatogenic stage is located in a different lobule (Figures 7 and 8).

During the reproductive cycle, each spermatogenic cell could dominate the testicular lobule, showing the occurrence of milt development and differentiation process [10]. According to [3], milt cells found in *Cyprinidae* fish during the development process had different characteristics, namely a vesicular nucleus with a nuclear membrane and nucleolus, more dominant primary spermatocytes than secondary spermatocytes, spermatids as the smallest cells with a solid nucleus and an acidophilic ring-shaped area in the cytoplasm, as well as flagellated cells with a round nucleus and dark.

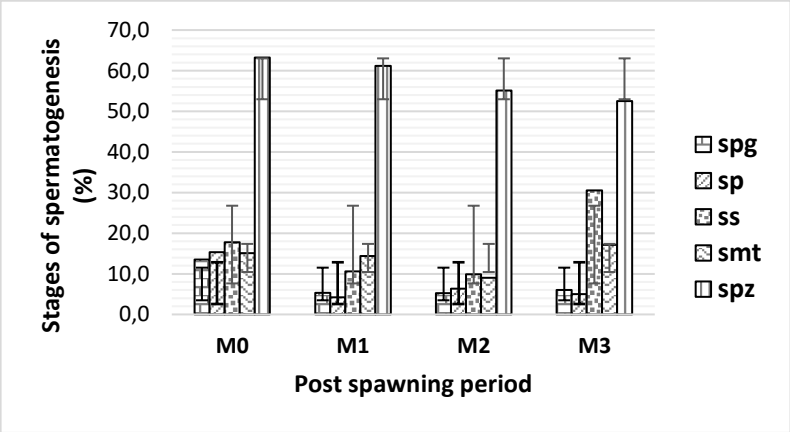


Figure 9. Proportion of each spermatogenesis stage in each post-spawning period of male freshwater Bawal

Based on Table 3 and Figure 9, the percentage of spermatogenesis stages (spz) produced from spermatogenesis process in freshwater Bawal after spawning from week 0 to week 3 remained relatively high. Therefore, freshwater Bawal consistently carried out spermatogenesis process after spawning until the next spawning.

Table 1 shows that after spawning, only 4 out of the 24 male freshwater Bawal could produce milt during striping (M0-1, M0-2, M2-4 and M3-4), while the others could not. The remaining 20 had GMI values between 2.48 - 4.02%, which according to [32] were male parents with matured gonads and ready for spawning.

This study was conducted from June to October 2023, when Banyumas and surrounding areas were in the dry season (≥ 14 hours of daylight). Studies in China (32) reported that the levels of FSHβ and luteinizing hormone (LH)β mRNA in the pituitary decreased during July (10 hours of light: 14 hours of darkness; spawning period). Therefore, photoperiod controlled the reproductive cycle of Chromis notata fish, mainly through its effects on reproductive hormones and IGS. The hormone profile of Teleost fish in sea and freshwater in various seasons was reported by [33]. However, only limited data were available on tropical fish. This study did not observe the hormonal profile of male freshwater Bawal during the post-spawning waiting period until the third week. The analysis of Bawal showed that testosterone and FSH levels increased from week 0 to week 3 after spawning. In general, fish from the Cyprinidae family, such as Osteochilus vittatus and Barbonymus gonionatus, as well as freshwater Bawal could lay eggs and produce milt in spawning season. Testosterone and FSH from male and female fish tended to stimulate gametogenesis until gonad maturation [34]. Also, E2 levels decreased

with an increase in progesterone, FSH, and testosterone levels until the subsequent spawning season.

Exposure to the dry season with a 14-hour photoperiod with almost no rainfall, as well as the process of moving mature gonad Bawal to a new location in this study, tended to cause stress. Fish experiencing stress often secrete more glucocorticoid hormones from the adrenal glands, inhibiting gonadotropin secretion from the pituitary gland [82]. Although in terms of progress, GMI value was significantly high to spawn (Table 1), it was unable to produce milt during stripping. Testis histological description also reached the spermatogenesis stage, having the highest percentage among the other stages of spermatogenesis (Table 3 and Figure 8). This was also observed in male Bawal adapting to the new environment in the natural cultivation pond from the original cultivation site.

Figure 9 shows the relative proportion of spermatogenesis stages in male Bawal during the post-spawning period of week 0 and week 1 (June to July; 12 hours of light: 12 hours of dark). The stage began to decline from July to October, namely the second post-spawning period and the third week (14 hours of dark). According to [33], GMI value was significantly lower when there was a short photoperiod (10 hours of light: 14 hours of dark) during spawning season. Histological analysis of the testis showed that the gonads of males in the 10 hours of light: 14 hours of dark and 12 hours of light: 12 hours of dark groups were in the 'resting stage', characterized by stable numbers of spermatogonia and perinuclear oocytes. Meanwhile, the 14 hours of light: 10 hours of dark group was in the maturing stage, characterized by mature milt (60%) (Figures 7 and 8).

## D. Conclusion

The following conclusions were drawn based on the results and discussions carried out:

Even though the data on the quality of sperm/milk is low, from histological observations, the process of gamete formation of male pomfret parents by the end of the research has on average reached the mature/sexually mature stage meaning that they are ready to mate/spawn. But during the dry season, Male Bawal is unable to ejaculate/release milt sperm. Maybe it's due to environmental stress factors and still needs further research.

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