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Research Article

The Gonadal Maturity and Gene Expressions of Female Giant Freshwater Prawn (*Macrobrachium rosenbergii*) after Dietary Administration of Medroxyprogesterone Acetate

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Abstract

One of the problems in giant prawn cultivation is female giant prawns laying eggs during rearing. They will incubate their eggs for three weeks so that the energy from the expected feed for growth is used for egg development and other reproductive activities. Giving MPA hormone to giant prawns either by injection or oral could inhibit gonadal maturation and increase growth rate. The maturation process of the prawn ovary consists of the activation of some complex cellular mechanisms involving genes that regulate the stages of oocyte development. This study aimed to evaluate the response of gonadal maturity and the expression of the *MrvWD-Kazal* gene in giant prawns fed with a diet containing MPA hormone. The design used in this study was a completely randomized design with four treatments and five replications. The treatments were feeding a diet added with MPA with a concentration of 0 $mg.kg^{-1}$ feed as control (K), 50 $mg.kg^{-1}$ feed (P1), 100 $mg.kg^{-1}$ feed (P2), and 150 $mg.kg^{-1}$ feed (P3). MPA hormone at a concentration of 50-150 mg/kg feed could inhibit the gonadal maturation of female prawns. In 100 mg/kg of feed concentration showed the lowest gene expression level, indicating an inhibition of gonadal maturation molecularly. The administration of MPA hormone through the feed is a recommended method of aquaculture because it is more applicable and effective than by injection, even though it has a non-uniform impact on each individual.

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1. Introduction

One of the problems in giant prawn cultivation is female giant prawns laying eggs during rearing. They will incubate their eggs for three weeks so that the energy from the expected feed for growth is used for egg development and other reproductive activities (Cavallo *et al*., 2001). This harms shrimp farmers because this reproductive activity can inhibit growth. Shrimp growth is influenced by sex, stadia, and several factors such as the amount of feed, feed quality, temperature, and water salinity (Dall *et al*., 1990). Efforts to increase growth performance can be made through breeding programs (selection and hybridization) and hormonal applications (Tave, 1995).

Inhibition of gonadal maturation can be done by using hormones, one of which is medroxyprogesterone acetate hormone (MPA), a synthetic progestin. Administration of MPA hormone to giant prawns either by injection or oral could inhibit gonadal maturation and increase growth rate (Wijaya *et al*., 2020; Anggraeni *et al*., 2021). Gonadal maturity level is closely correlated with gonadosomatic index (GSI), oocyte diameter, and gonadal histological characteristics (Chang and Shih, 1995). The gonadosomatic index is the main sign of gonadal development. Naturally, the GSI means bonylip barb in Lake Tamblingan always increases with increasing gonad maturity level, except for gonad maturity lebel V where the fish spawned (Parawangsa *et al*., 2022). It gradually increased during the trial period with serotonin injection. The GSI value in the control was 2.9 ± 0.04 and increased to 3.88 ± 0.88 with serotonin injection, while GSI did not increase with dopamine injection (2±0.03) (Aprajita *et al*., 2014). Previous studies reported that administering MPA and dydrogesterone (DDG) in zebrafish at concentrations of 4.5 – 4.8 ng/L could induce several transcriptional responses and, at higher concentrations, could affect reproduction and gonadal histology (Zhao *et al*., 2015).

Moreover, the maturation process of the prawn ovary consists of the activation of some complex cellular mechanisms involving genes that regulate the stages of oocyte development (Qiu and Yamano, 2005). Genes associated with the ovarian maturation process of *M. rosenbergii* are not much known compared to those involved in the reproductive of *Macrobrachium nipponensis*. *M. nipponensis* has five reproductive genes that are known to be related to ovarian development, namely Mago-nashi (MnMago), Tsunagi (MnTsu), Gustavus, Ubc9 (MnUbc9), and von Willebrand factor D-Kazal (MnvWD-Kazal) (Zhang *et al*., 2010, 2011a, 2011b, 2016). One of the genes studied in giant freshwater prawns (*M. rosenbergii*) involved in ovarian maturation is von Willebrand factor D (vWD) – Kazal. During the maturation process, the oocyte membrane receptor binding to vitellogenin is carried out by the vWD (Baker, 1988). The expression of the *vWD-Kazal* gene has been studied in female giant prawns called *MrvWD-Kazal* (Faiz *et al*., 2019). During the gonadal maturation stage, the relative abundance of MrvWD-Kazal mRNA in female giant prawns changed in contrast to the relatively unchanged relative abundance of this gene in the stomach and intestine (Faiz *et al*., 2019).

However, all previous studies were focused on the effect of medroxyprogesterone acetate on physical parameters such as inhibiting gonadal maturation, increasing the growth of female giant prawns, and the survival rate of the broodstock (Anggraeni *et al*., 2021). To authors' knowledge, very few studies have been conducted concerning on the *vWD-Kazal* gene expression of female giant freshwater prawns and a more in-depth discussion of gonad maturity response. Therefore, this study aims to evaluate the response of gonadal maturity in gonadal histology and the gene expression of the *MrvWD-Kazal* gene in giant freshwater prawns fed with a diet containing MPA hormone.

2. Materials and Methods

2.1 Materials

The study was carried out from September 2020 to May 2021 at the hatchery and Physiology & Genetics Laboratory of the Research Institute for Fish Breeding (RIFB), Patokbeusi, Subang, West Java, Indonesia. Gonadal histology was prepared in the Fish Health Laboratory Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, and then analyzed in the Microbiology Laboratory of RIFB. The giant freshwater prawn used was a female strain G-I Macro-II breeding resulted from the Research Center Fish Breeding (Khasani *et al*., 2018) which were five months old, 9.66 ± 1.48 g weight, healthy, and had complete organs.

2.2 Methods

2.2.1 Experimental design

Research on maturity responses and gene expressions of female giant freshwater prawns were conducted using experimental methods. The design used in this study was a completely randomized design (CRD) with four treatments and five replications. Twenty aquarium units equipped with water filters were used in this study. This aquarium was equipped with a screen made of wire to avoid prawn cannibalism. Stocking density of ten prawns per aquarium and

was acclimatized for three days. The treatments were feeding diets added with MPA with a concentration of 0 mg.kg^{-1} feed as control (K), 50 mg.kg^{-1} feed (P1), 100 mg.kg^{-1} feed (P2), and 150 mg.kg^{-1} feed (P3). During the experiment, the giant freshwater prawns were fed according to the treatment with a feeding rate of 3% per day of the biomass. The treatment lasted for 60 days. Observations of the gonad maturity level were carried out once a week.

2.2.2 Gonad maturity observation

Observation of the level of gonadal maturity was carried out once a week by observing the development of the gonads visually from the color of the ovaries which are located on the cephalothorax based on Reddy *et al.* (2013).

2.2.3 Gonadosomatic index and gonadal histology

Observation of the gonadosomatic index was carried out at the initial and the end of study with the equation below:

$$GSI = \frac{Weight\ of\ the\ gonad}{Weight\ of\ the\ prawn} x\ 100 \quad \ldots \text{Eq 1}$$

Observation of the histological preparations of the gonads was carried out to evaluate the histological structure microscopically. The samples used in this study were gonads of 10 prawns at the initial and end of the experiments, taken from each treatment, namely 0 mg.kg feed^{-1} as the control group, 50 mg.kg feed^{-1}, 100 mg.kg feed^{-1}, and 150 mg.kg feed^{-1}. The parameter observed in this study was oocyte morphology to see the level of gonad maturity. Gonad preparations were made using the Hematoxylin-Eosin (H&E) staining method (da Silva *et al.*, 2009). Prawns were dissected, and the gonads were isolated and then fixed in Davidson's AFA solution for 24 hours. The fixed gonads were transferred to 70% alcohol to start the dehydration stage. Histological observations of the gonads and image files were taken using a binocular microscope connected to a computer and were displayed on the monitor using the analysis software program. The resulting data were analyzed descriptively.

2.2.4 Gene expression analysis

The first step of gene expression analysis was collecting the samples of giant freshwater prawns treated with different concentrations of medroxyprogesterone acetate (MPA) hormone. The gonad samples were taken at the end of treatment with different concentrations as described previously, and the organs were then stored at -80°C until ready for use. The RNA extraction from the gonad was carried out by taking approximately 50 mg from each treatment and then isolated using Quick RNATM mini prep plus (Zymo Research, American). Furthermore, the concentration of RNA obtained was measured with the RNA copy number calculator program (Qubit) to obtain a standard concentration. In this study, a one-step kit was used to combine the complementary DNA synthesis (cDNA) process with the gene expression level quantification process. The following level became quantitative PCR (qPCR) reactions of MrvWD-Kazal and housekeeping (beta-actin) genes related to reproduction using primers by Faiz *et al.* (2019) and Mohamad *et al.* (2017) (Table 1).

Quantifying the relative expression of the goal gene transcript with the reference gene used the Rotor-Gene Q Thermocycler series software program technique. Master mix SensiFAST SYBER No-ROX one-step kit (Bioline) was used as a real-time PCR reagent with a volume of 20 µL of reaction. The temperature profile for one-step SensiFAST SYBER No-ROX was reverse transcription of 3-step cycling was 45°C for 10 minutes, then polymerase activation was 95°C for two minutes, followed by denaturation was 40 cycles at 95°C for five seconds, and annealing was 60°C for 10 seconds, and the end step was an extension at 72°C for five seconds. The MrvWD-Kazal and beta-actin curves were created at the quilt of qPCR (melting curve effects are furnished inside the quick statistics). Amplification data were analyzed using the 2 -ΔΔCT method (Livak and Schmittgen, 2001).

3. Result and Discussion

3.1 Gonadal Maturity Response

Gonad maturity level indicates a level of prawns' sexual maturity. The level of gonad maturity is classified into five classes based on histologic features, namely stage 0 (spawn/laying eggs), stage I (spent), stage II (proliferative), stage III (premature), and stage IV (mature) (Reddy *et al.*, 2013). The process of vitellogenesis or gonad maturation based on visual observation consists of two stages, namely immature (immature gonads) and mature (mature gonads). The color of the ovary appears transparent at the immature stage (stage I and II), and when the vitellogenesis process reaches the mature stage, the color of the ovary changes from orange to dark brown (stage III and IV) (Nagaraju, 2011). The effect of feeding diets containing MPA hormone on the level of gonadal maturity showed a higher percentage of immature prawn compared to the

control at the sixth week (Figure 1). Administration of MPA to giant prawns at a concentration of 100 mg/kg of feed was able to inhibit the reproductive stage of prawn until the fourth week by 100%, while the administration of MPA at concentrations of 50 mg/kg and 150 mg/kg of feed and control was only able to inhibit the fourth week by 82.5%, 86%, and 81.7%, respectively.

The treatment of diets containing MPA hormone at feed concentration of 100 mg/kg decreased in the fifth week, but in the sixth and seventh week, it tended to stagnate and decreased again in the eighth week. The same pattern occurred in the treatment of feeding diets containing MPA hormone at feed concentrations of 50 and 150 mg/kg, where there was a decrease in the third week then stagnation and a decrease again at the end of the study. The patterns in the treated group were in contrast with the control treatment which continued to experience a decrease in the percentage of immature prawns until the end of the treatment. The difference is thought to be due to the presence of additional feed additives, namely MPA hormone accumulated in the prawn body. The administration of MPA to giant freshwater prawns at a feed concentration of 100 mg/kg was able to maintain the percentage of immature prawn until the end of the study by 78.6%, while at feed concentration of 150 mg/kg, 50 mg/kg, and control treatment by 70.2%, 69.4%, and 57.3%, respectively. It was shown that administration of MPA hormone to giant freshwater prawns at feed concentrations of 50, 100, and 150 mg/kg could inhibit gonad maturity significantly ($p<0,05$).

Table 1. The primer used in qPCR of MrvWD-Kazal gene.

No.	Primer	Sequence (5'-3')	Reference
1.	FZ-RTF	ATGGAAGAGCATCTTGTCTGAG	Faiz *et al.* (2019)
	FZ-RTR	TCCATTCACTGTATAACTGGAAGTC	
2.	Beta-actin F	CGTGACATCAAGGAGAAGCTGTG	Mohamad *et al.* (2017)
	Beta-actin R	TGACCGTCGGGGAAGCTC	

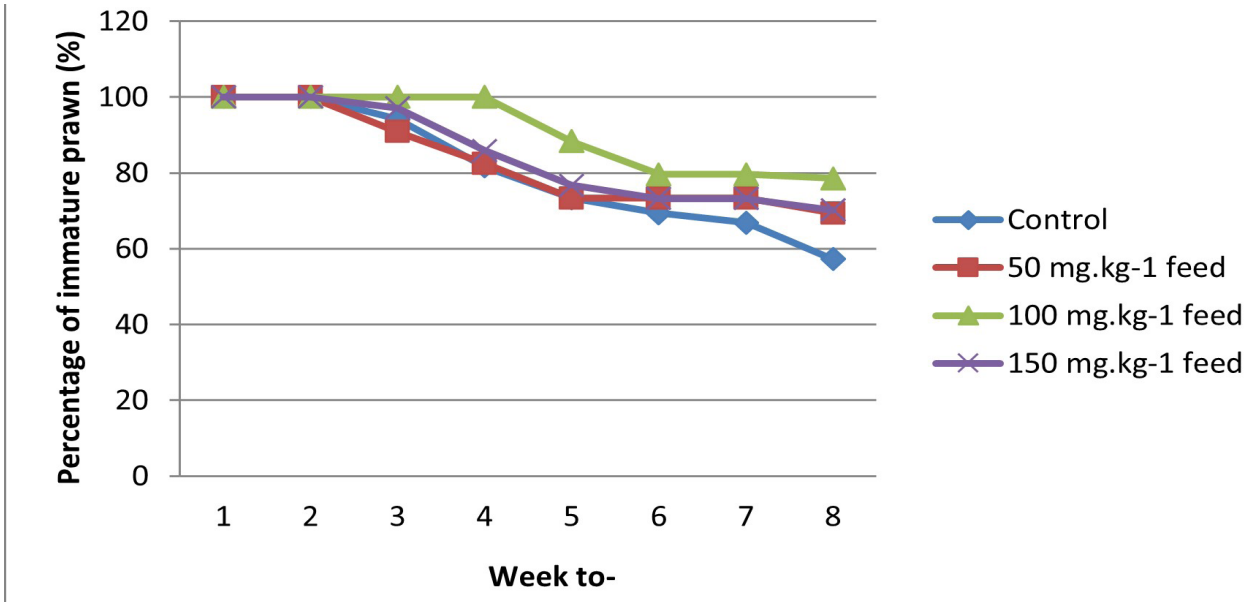

Figure 1. Percentage of immature gonads of giant freshwater prawns in each treatment during the study; control (MPA concentration of 0 mg.kg^{-1} feed), MPA concentration 50 mg.kg^{-1} feed (P1), MPA concentration 100 mg.kg^{-1} feed (P2), and MPA concentration150 mg.kg^{-1} feed (P3). Data shown as means ± SD (standard deviation) for five replications. Different lowercase letters (a, and b) indicated difference ($p<0,05$); same lowercase letters indicate no significant difference in the treatments.

The level of inhibition of MPA hormone through feed was fluctuating on every prawn. In general, the administration of MPA hormone can inhibit gonadal maturity as indicated by a high percentage of immature prawns. However, the impact was not the same for every prawn, which apparently was influenced by the ingested portion of hormone-containing feed (Figure 2). In the control treatment, the percentage of fully matured prawns was 42.7%, prawns that entered the maturation period was 13.5%, while prawns treated with MPA hormone, only 2.5-5% was in renaturation stage. From the results of this study, even though oral hormone application is less effective than injection, it is still more efficient on a commercial scale because of the easy administration technique and does not have a stressful impact on prawn. This is in line with the research of Laining *et al.* (2015) who stated that the hormone application technique by injection is more effective, but requires more expertise, especially in minimizing the stress level of prawn during the injection. Meanwhile, oral administration of hormones is considered to be more efficient in mass application, although higher doses are required.

Gonad maturity level is closely related to gonadosomatic index (GSI), oocyte diameter, and gonadal histological characteristics (Chang and Shih, 1995). The gonadal maturity index is the main sign of gonadal development. The gonadosomatic index (GSI) of giant freshwater prawns at the beginning of the study was not significantly different ($p>0.05$) among treatments. There was an increase in the gonadal maturity index at the end of the study. The value of gonadal maturity index of prawns fed diets containing MPA hormone was lower than the control (Figure 3). However, this study could not be analyzed using statistics. The gonadosomatic index values between immature and mature shrimp have a very wide range, causing the data to be inhomogeneous. The immature gonadosomatic index values were 0.12-0.60, while the immature gonadosomatic index values were 4.13-5.46. The increase of GSI value at the end of maintenance indicated a development of gonadal maturity in each treatment. The GSI values of P1, P2, and P3 treatments at the end of the study were lower than GSI values in the controls because of the influence of MPA

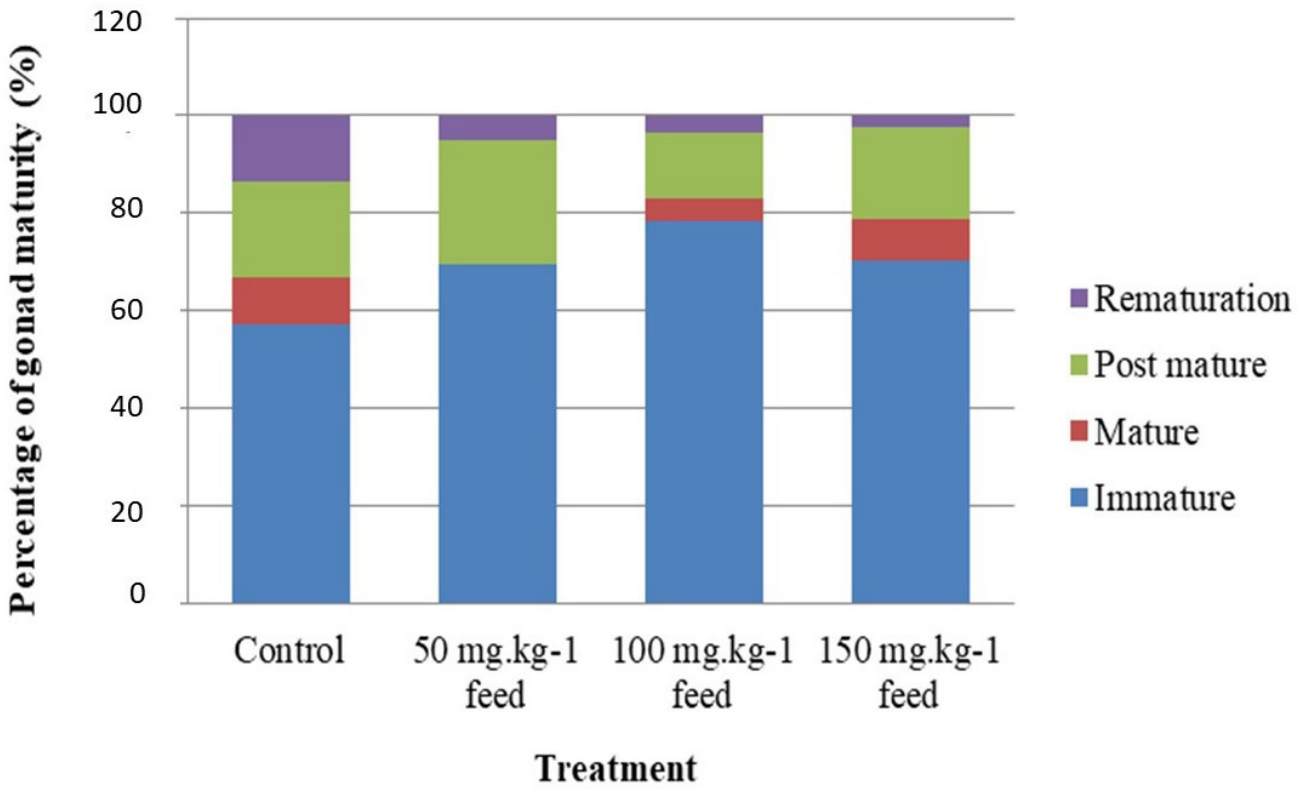

Figure 2. Percentage of gonadal maturity levels at the end of the study in each treatment; control (MPA concentration of 0 mg.kg-1 feed), MPA concentration 50 mg.kg-1 feed (P1), MPA concentration 100 mg.kg-1 feed (P2), and MPA concentration150 mg.kg-1 feed (P3)

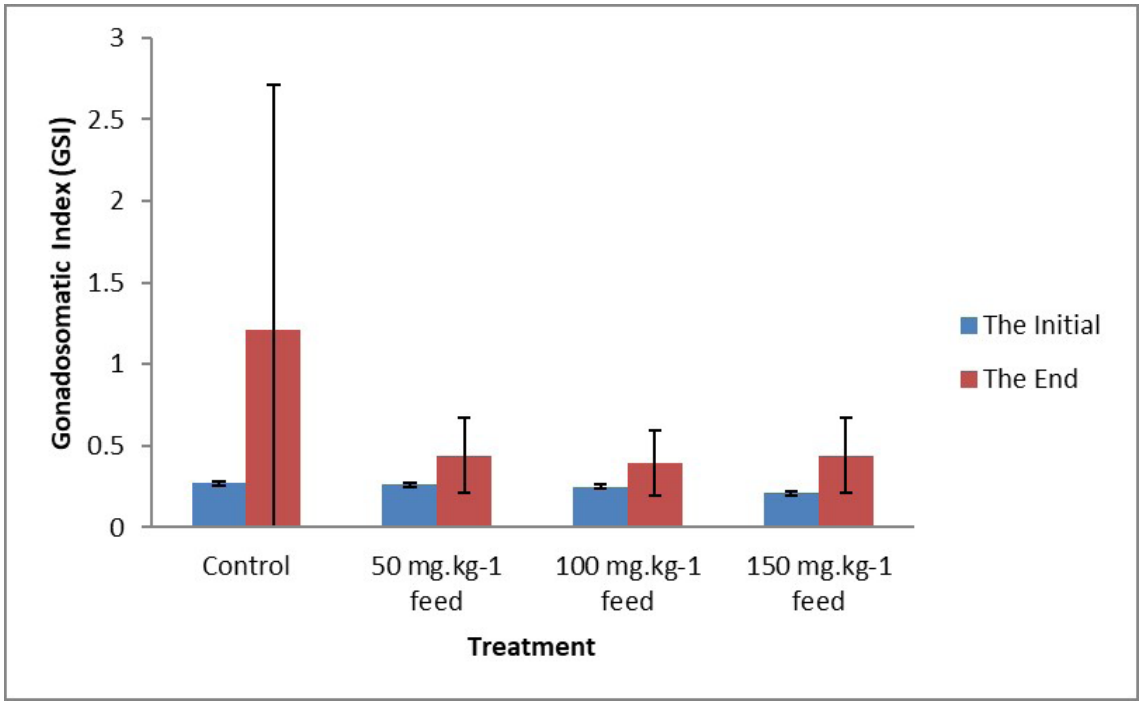

Figure 3. Gonadosomatic index (GSI) of giant freshwater prawns at the initial and the end of the study

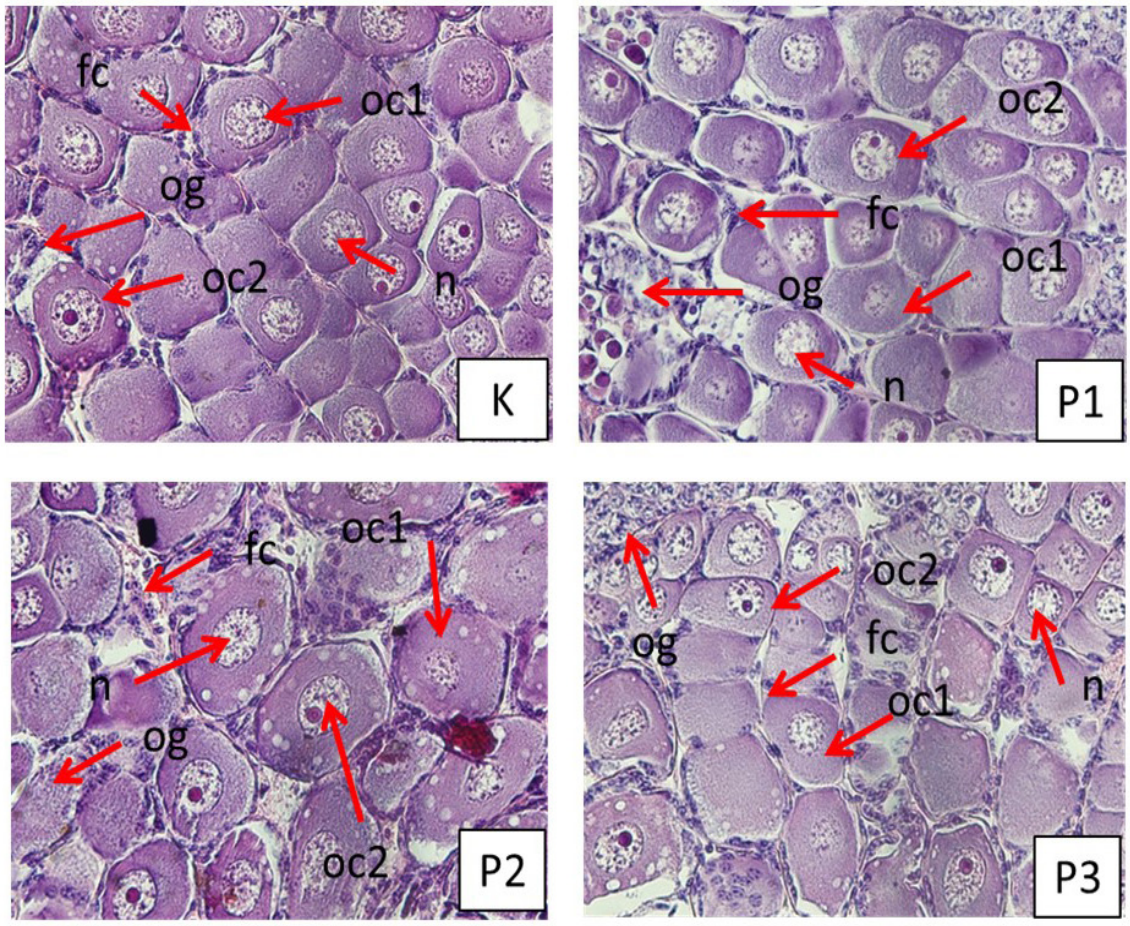

Figure 4. Histology of giant freshwater prawn gonads before treatment (400 times magnification) with hematoxylin and eosin staining. Note: n: nucleus, Fc: follicular cell, Og: oogonia, Oc1 & Oc2: previtellogenic oocyte

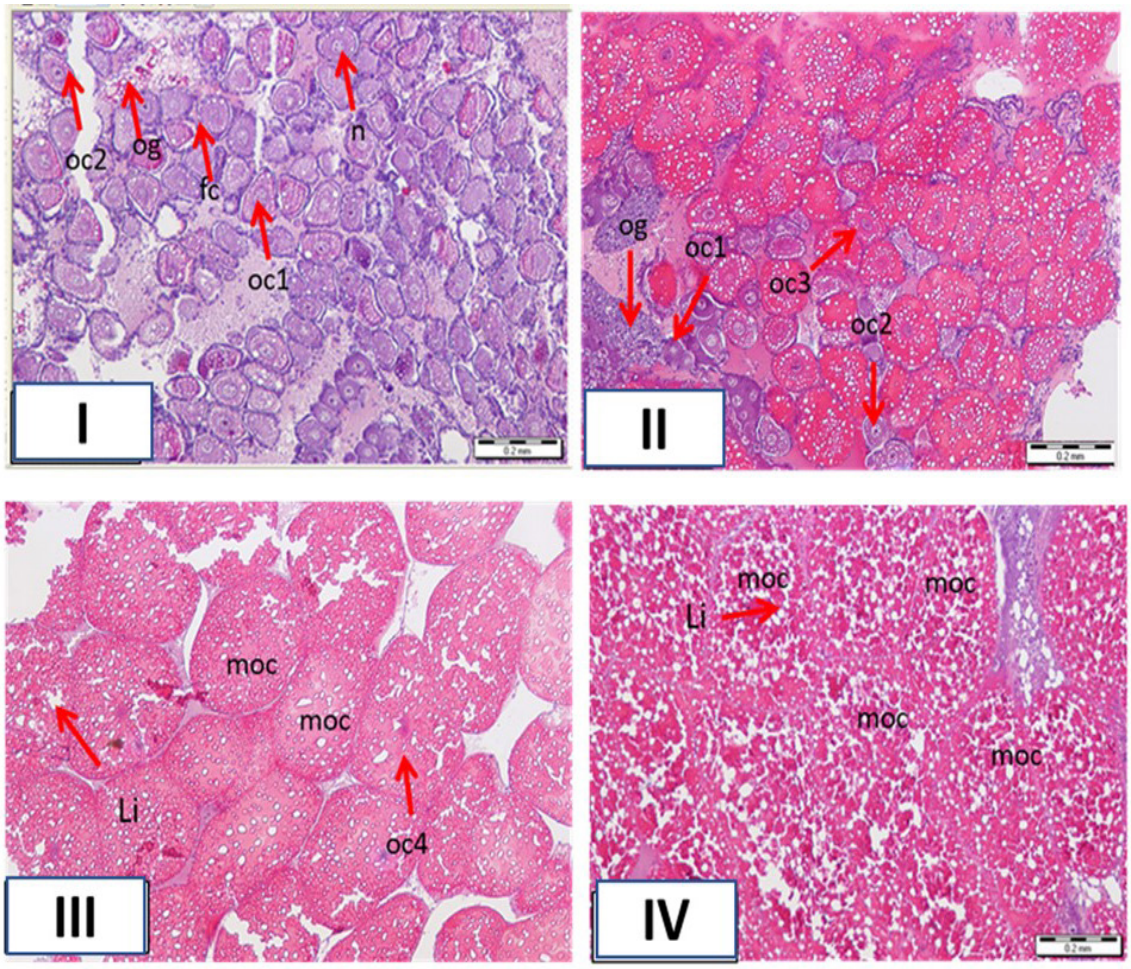

Figure 5. Histology of gonads after treatment (100 times magnification) with hematoxylin and eosin staining. Note: n: nucleus, Fc: follicular cell, Li: lipid droplet, Og: oogonia, Oc1 & Oc2: previtellogenic oocyte, Oc3: early vitellogenic oocyte, Oc4: late vittelogenic oocyte, moc: mature oocyte (Reddy *et al*., 2013)

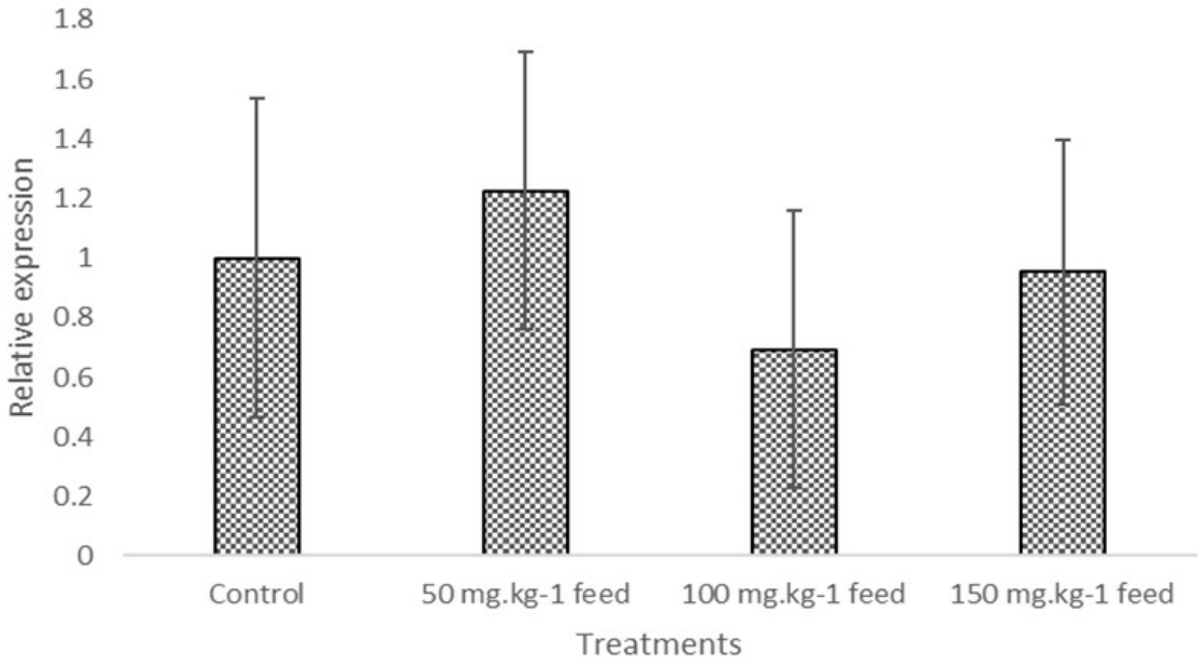

Figure 6. MrvWD-Kazal gene expression level at the end of the study. The value of gene expression was relative to control Y-axis which represents the relative mRNA transcript abundance of MrvWD-Kazal/ β-actin

hormone which inhibited gonadal maturation. The control treatment had a higher GSI value because the percentage of gonadal maturity of prawns in the control treatment was higher. The GSI value will increase in line with the development of the gonadal maturity level. The higher the level of gonadal maturity, the higher the GSI value (Reddy *et al*., 2013). The results of the same study stated by Okumura (2004) showed that the level of vitellogenin in hemolymph increased when the GSI value increased. The increase of GSI value from each increase in the level of gonadal maturity is doubled (Chang and Shih, 1995). The relationship between body weight and GSI with gonad maturity for female prawn was significant ($P<0.05$), where the increase in GSI is also influenced by the increase in maturity stage due to an increase in the gonad volume after reaching the mature stage (Fatihah *et al.,* 2022). However, in this study, the uniformity of gonadal maturity levels in each individual in each treatment was low so that the GSI deviation in each treatment was quite high (Figure 2). Determination level of gonadal maturity can be done in two ways; observation based on general signs and gonadal size and microscopic observation through the histological picture of the gonads (Effendie, 1979). The development of gonadal maturity level in giant freshwater prawns consists of five stages which are characterized by changes in oocyte color and size (Chang and Shih, 1995; Reddy *et al*., 2013). At the beginning of the study (before given feed containing MPA hormone), the giant freshwater prawns' gonads in each treatment were at stage I of gonadal maturity level (Figure 5). The gonadal maturity level stage I is the previtellogenesis stage which mostly consists of oogenia and previtellogenic oocytes (oc1 and oc2) and the surrounding follicular cells. The ovaries in stage I contain previtellogenic oocytes (oc1 to oc2) and follicular cells (Reddy *et al*., 2013).

The effect of feeding diets containing MPA hormone on prawn gonad histology for 60 days showed the development of different levels of gonad maturity in each treatment. Each treatment had levels of gonadal maturity stages I, II, III and IV. The gonadal maturity level stage I showed the gonadal cells development at the previtellogenic stage (oc1 and oc2), and the cell size looked smaller. The gonadal maturity level stage II showed gonadal development at oc3, a small portion of oc1 and oc2, and still visible oogonia. Gonadal development at stage III was at oc4, and some began at the mature stage (moc). While at stage IV, the development of the gonads was at the mature stage (mature oocyte/ moc). The size of mature oocyte at stage IV was larger, and it was seen that each cell was not separated from the other compared to the mature oocyte at stage III. At stages III and IV, follicular cells did not appear to be enlarged. This was due to the stretching of the cytoplasm caused by intense oocyte growth. The increase in oocyte diameter occurs in gonad development from stage I to stage IV (Reddy *et al*., 2013).

The histological preparations of these gonads used hematoxylin and eosin, but the results showed different colors. The picture of the gonads before treatment showed a purple color, as well as the histological picture of the gonads at the stage I after being given treatment. This is because, at the previtellogenic stage, gonadal cells are more basophilic than in the vitellogenic stage, so they absorb the purple color more (Chang and Shih, 1995).

3.2 Gene Expression Analysis

Gene expression is the process of using genetic information stored in genes to synthesize compounds in the form of proteins. The effect of feeding diets containing MPA on reproductive performance can be seen molecularly from the expression of the MrvWD-Kazal gene. The results of gene expression analysis showed that P2 and P3 treatments were 30% and 10% lower, respectively, than the control treatment. However, P1 treatment showed higher gene expression, which was presumably due to the high variation in gene expression values in each prawn. The P2 treatment showed the lowest gene expression level. The low expression of MrvWD-Kazal gene in treatments P2 and P3 indicated molecularly that inhibition of gonadal maturation using MPA hormone through feed concentrations of 100 and 150 mg/kg was able to inhibit gonadal maturity in female giant prawns (Figure 6). This is in line with the results of research by Faiz *et al*. (2019), stated that the expression of MrvWD-Kazal gene in the ovaries changes during the ovarian maturation stage. The expression of MrvWD-Kazal gene at stage II increased 3.5 times compared to stage I. At stage III, the expression of MrvWD-Kazal gene was the highest, which was six times compared to the expression of the gene at stage I, while the expression of the gene at stage IV decreased and only 1.2 times of stage I (Faiz *et al*., 2019).

Most of the gonad maturity levels in P2 and P3 treatments in this study were at the immature stages, thus lowering the expression of MrvWD-Kazal gene's expression. The von Willebrand factor D (vWD) – Kazal gene that has been studied in the genus Macrobrachium is a gene involved in gonadal maturation (Faiz *et al*., 2019). The vWD protein functions within the binding of oocyte membrane receptors to vitellogenin and its absorption in oocytes (Baker, 1988). The vWD domain provides sites to facilitate the binding of vitellogenin

to its vitellogenin receptors (Finn, 2007). The vWD protein has properties and functions as an adhesive for the oocyte membrane receptor. Steroid hormones usually bind to a protein receptor in the cytoplasm and move into the nucleus, there is an effect that affects genetic mechanisms (Tata and Smith, 1979). Therefore, diets containing MPA hormone are thought to affect the gonadal maturation process that will have an impact on the genetic mechanism shown by the expression of MrvWD-Kazal gene. In tiger shrimp (*Penaeus monodon*), simultaneous expression of estrogen and progesterone receptors in the ovaries was found, adding further evidence that steroid hormones mediate the expression of the vitellogenin gene in Penaeid shrimp. Thus, it was shown that estradiol and progesterone control the transcriptional activation of the vitellogenin gene in crustaceans (Merlin *et al*., 2015).

The results of the study on the expression of von Willebrand factor D (vWD) – Kazal gene had previously been studied in another genus of Macrobrachium, namely *M. niponnensis*. The mRNA expression of MnvWD-Kazal gene within the ovaries gradually elevated from the perinucleolus (PN) degree to the yolk granule degree (YG) and reduced in the maturation stage (MA). The most expression occurs on the YG degree, while the least expression occurs on the late paracmasis (PM) or vitellogenin degree. Variations inside the expression of MnvWD-Kazal at diverse degrees of ovarian development suggest that this novel gene performs a critical position in *M. nipponense* oocyte maturation (Zhang *et al*., 2011a).

4. Conclusion

This research evaluated the response of gonadal maturity in gonadal histology and the gene expression of von Willebrand factor D *(vWD) – Kazal* gene in female giant freshwater prawns fed with a diet containing Medroxyprogesterone acetate hormone. MPA hormone at a feed concentration of 50-150 mg/kg could inhibit gonadal maturation of female prawns. In 100 mg/kg, feed concentration showed the lowest gene expression level indicating the inhibition of gonadal maturation molecularly. The administration of MPA hormone through the feed is a recommended method for aquaculture because it is more applicable and effective to inhibit the early maturation of prawns.

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Authors' Contributions

The contribution of each author is as follows, FA; plotted and conducted the research, collected the data, designed the figures and tables, finalized the manuscript, analyzed gene expression, drafted the manuscript. KS: supervised the gene expression analysis. II and DMM; evaluated the final data and did critical revisions of the manuscript. JH, IK, AS; involved in supervising hatchery work. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest. We certify that the submission is original work and is not under review at any other publication.

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