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The Gonad Maturity of Female *Osteochillus vittatus* in the presence of Ascorbic Acid

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Abstract

The fish's reproduction status is affected by both fed compositions and vitamins intake lead to determine improvement of eggs quality as well as fish production. The presence of Ascorbic Acid (AA) in the cultivation ponds, might accelerate female's gonad maturation and so rematuration. The research aimed to determine: (1) Level gonad maturity of supplementation Ascorbic Acid; (2) Oocyts diameter; (3) Larvae survival rate. The research used experimental methods.The method was a completely randomized design (CRD) of 4 treatments and 4 replications. Treatments were supplementation of AA at different dosages of 0, 600, 1200 and 1800 mg/kg fish-fed ration.The gonad maturity level was analysed descriptively and oocyte diameter and larvae survival rate data were analysed by ANOVA. The result showed that supplementation of AA at a dosage of 1200 mg / kg fish-fed ration accelerated the process of gonad maturity, development of oocyt diameter and larvae survival rate as well as the viability of *O. vittatus* larvae at 90 rearing days. In this case, level IV gonad maturity was reached at 90 days which characterized by completed vitelogenesis process and oocyte diameter of 1.1 mm. Thus, this study is useful for aquaculture science by providing information on utilization of AA as food supplement in fish culture and also for fish farmer who wish to accelerate *O. vittatus* reproduction.

How to Cite

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INTRODUCTION

Gonads maturity of adult female fish is determined by either environmental or genetic aspects. Nutrition as well as fed components like fats, amino acids, minerals, and vitamins might also play significant role in affecting the fish reproduction phase) (Canyurt & Akhan, 2008). Vitamin C (Ascorbic Acid/AA), one among those micro nutrients, supposed to present in the fish fed, since this compound has significant role on fish reproductive status and must be present in the fed since they do not have these in nature. The absence of L-gulonactone oxidase enzyme in teleost fish, which catalyses AA from sugar, causes the fish are not able to synthesis AA. The AA therefore needs to be added on on the feeding rations of this fish (Ibrahem et al., 2010) whereas, the third one received a balanced diet supplemented with vitamin C (500 mg kg-1 group since this component takes a significant role in optimallizing the fish reproductive performance especially during vitellogenesis as well as embryo development. Supplementation of AA on the fish-fed ration during ovum development in the ovarium may increase the ova hatching rate and fish larvae development (Sandnes, 1991).

Several studies on the supplementation of AA on the fish-fed ration related with its effect to the gonad maturitiy of Cod (*Gadus morhua* L) and sperms quality of the Trout (*Oncorhynchus mykiss*) had been reported previously (Canyurt & Akhan, 2008). In the last group of fish, Trout (*Oncorhynchus mykiss*), it was noted that AA addition on fed-ration to be an important factor of its reproductive performance especially when they reached the maturity status (Valdebenito et al., 2015) both male and female, are determined by several factors (age, management, feeding, chemical and physical factors, water quality, etc.. Additonal AA on the catfish (*Clarias gariepinus*) ration accelerated its gonad maturity on level III, i.e.: accelerating the oocytes stadia (Hengky, 2010). Other vitamins, like vitamin C, E an Zn mineral on the Nile tilapia (*O. niloticus*) ration might improve spawning frequency, larvae productions, fecundity, hatching rate, sperm motility and viability (Gamanpilla et al., 2007). During the study of reproductive performance, vitamin C (AA) supplemetned at a dosage of 150 mg kg-1 on the *O.niloticus* fed ration incrase the Gonado Somato Indeks (GSI) (Martins et al., 2016). Gonad maturity is related to egg development. Sandnes reported, that AA supplementation on the fed ration of salmon fish resulted a variable increase of egg diameter (Sandnes, 1991). Different habitat was also noted to cause different egg size, since the diferent habitat may contain different levels of nutrients on their natural fed of *Channa lucius* (Syandri, 2013) and *O. vittatus* (Syandri & Azrita, 2015). Disuniformity of mature egg diameter might be used to determine the spawning frequency as well as spawning period by checking the modus on the eggs diameter (Sharifuddin & Omar, 2010).

Nilem fish *(Osteochillus vittatus*, Cyprinidae) is a native Indonesian fish and has high in economic value (Kottelat et al., 1996). Naturally, the gonad development of this fish group is strongly depending on its habitat leads to a very limited frequency of spawning to twice per annum. Spawning frequency of the nilem, however, might happen monthly though it will reach maximum spawning frequency on a particular month on the year (Subagja et al., 2006). Increasing spawning frequency which does not always depend on this environmental nutrients content, has alredy been applied in the fish-cultivation practices by increasing nutrition on the fish-fed ration and additional supplement toward the female fish. Addition of 30% protein and supplementation of ascorbic acid (AA) reported to improve reproductive performance of *O vittatus* to reach its gonad maturity and rematuraty earlier than normal condition. By doing so, it is expected that spawning frequency of the *O vittatus* might happens more than two times per annuum leads to increase production of fish larvae, especially for fish famers.

The aim of the research was to level gonad maturity of supplementation Ascorbic Acid, oocyts diameter and larvae survival rate.

METHODS

Experimental materials 64 adult individuals of post-spawning females *O.vittatus* taken from the Banyumas Regency Central Java, starch of ascorbic acid (AA), fish-fed ration contain 30% protein, *Tubifex* worms, Neutral Buffer Formalin (NBF) 10% solution, tapioca starch, aquabidest, synthetic hormon (Ovaprim).

All adult individuals of females *O.vitttus* were grown separately in 16 different ponds (1x2x0,5m/pond), each pond was filled with 4 individuals of tagged-fish. The fish were fed by ration supplemented with AA as on the treatment dosages i.e.:3% of their total body size and applied twice per day, morning and afternoon.

Current research was done experimentally by applying a Completely Randomised Design (CRD) with four treatments performed in four replications. Treatments were supplementation

of AA toward adult individuals of post spawning female *O.vittatus* at different dosages of 0, 600, 1200 and 1800 mg/kg fish-fed ration (Hengky, 2010). The observed variables were: gonad maturity, oocyt diameter, and larval viability. The adult female-fish were reared for total of 90 days.

Observation was done by dissecting the adult female fish at the age of 30 , 60 and 90 days of rearing, then observed for its histological appearance. Oocyt's diameter was observed by applying canulation method i.e.: by taking the ovum using a particular hose that fit to the genital size and pulled by a spuit (Setyaningrum et al., 2006). Fifty eggs were observed for their diameter under oculer microscope completed with micrometer. Sampling was done in the interval of 2 weeks, when the oocyt's size reaches 1.1 mm diameter means that the fish has ready to be spawned. Artificial spawning was done by applying synthetic hormon namely ovaprim.

Larvae of the hatched eggs were then grown in a fish aquarium compeleted with aeration system, on each treatment 100 larvaes were reared and performed in four replicates. Larvae were subjectyed to be fed naturally contains tubifex worms, and observed for its viability in 10 days of rearing.

Samples which were gonad maturity observation were taken at the interval for 30 days of rearing for 90 days, but the oocyt diameter was observed every two weeks. Samples were then put in the preparat flasks and added by 10% NBF solution, shook homogently to be ready for histological preparation and some were subjected for measurement of their size under ocular microscope completed with micrometer. The larvae viability was determined by counting total number of dead-larvae after 10 days.

Measurement of ovum diameter = total ocular scales multiplied by calibration value (Effendie, 1979).

Searching for 1 ocular value on the objective scale:

1 ocular x total oculars= total objectives x objective

1 oculars = Larvae's viability: Survival (%) = % (Effendie, 1979)

Data of gonad's maturity were analysed descriptively as done by Hibiya(Hibiya, 1982). Data of oocyt diameter and larvae viability were analysed statistically by applying ANOVA on a one way analysis using an SPSS version 21 software, when there was a significant different ($P<0,05$) analyses was then continued by test of least significant difference (LSD) test for determining oocyt diameter in relation to the the best larval viability.

RESULTS AND DISCUSSION

Gonad Maturity

At the rearing period of 30 days, gonad maturity of *O. vittatus* fish reached level III with two different stages of yolk formations i.e.: previtellogenesis which dominated by appearance of primary oocyte (op) which characterised by globular yolk (yg), and vitellogenesis stage which determined by the formation of secondary oocytes (os) but in small numbers. The OS consists of yolk plate (yp) (Figure 1). In all treatments, the study noted that gonad's maturity was dominated with the presence of primary oocytes and the nucleus which was located in the center of the ovum. Treatments of AA of 1200 mg/kg and 1800 mg/kg fish fed produced many secondary oocytes, and nucleus was in the postition of going to the edge of the cell.

A histological observation of the *O. vittatus* fish gonad after 30 days rearing showed if the both types oocytes (primary and secondary) had started to develop, as determined by the presence of globular yolk (yg) so it can be said that the ovarium was still in a pre-vitelogenesis (level III) stage. At this stage, the primary oocytes were still has its vacuoles and globular yolk, however there were some secondary oocytes appeared to sign that the gonad has almost in mature phase (Syandri & Azrita, 2015); (Ritonga et al., 2016). The finding of high number of primary oocytes shows the early phase of gonad maturity (Mannan, et al, 2010). Dorostghoal et al has as noted that there were three different phases of gonad maturity in the *Barbus grypus* namely: previtellogenic, vitellogenic, and maturation. The first phase was divided into two phases: primary oocytes and globular yolk; the vitellogenic however divided into three different stages; primary, secondary and tertiary globules. The last period, maturation, is characterized by two phases migration of the nucleus and hydration (Dopeikar et al., 2015).

Gonad maturity after 90 days of rearing period was characterized by its ovum which had reached the vitellogenesis where the secondary oocytes (os) were determinant. Apart from that, it was obviously seen if the nucleus almost reached the Germinal Vesicle Breakdown (GVBD) stage and so in periphere site (Figure 2). At either the absence of AA or 600 mg/kg fish-fed ration, current study noted if primary oocytes (op) were still present, however, treatment of AA supplementation at both 1200 mg/kg and 1800 mg/kg fish-fed

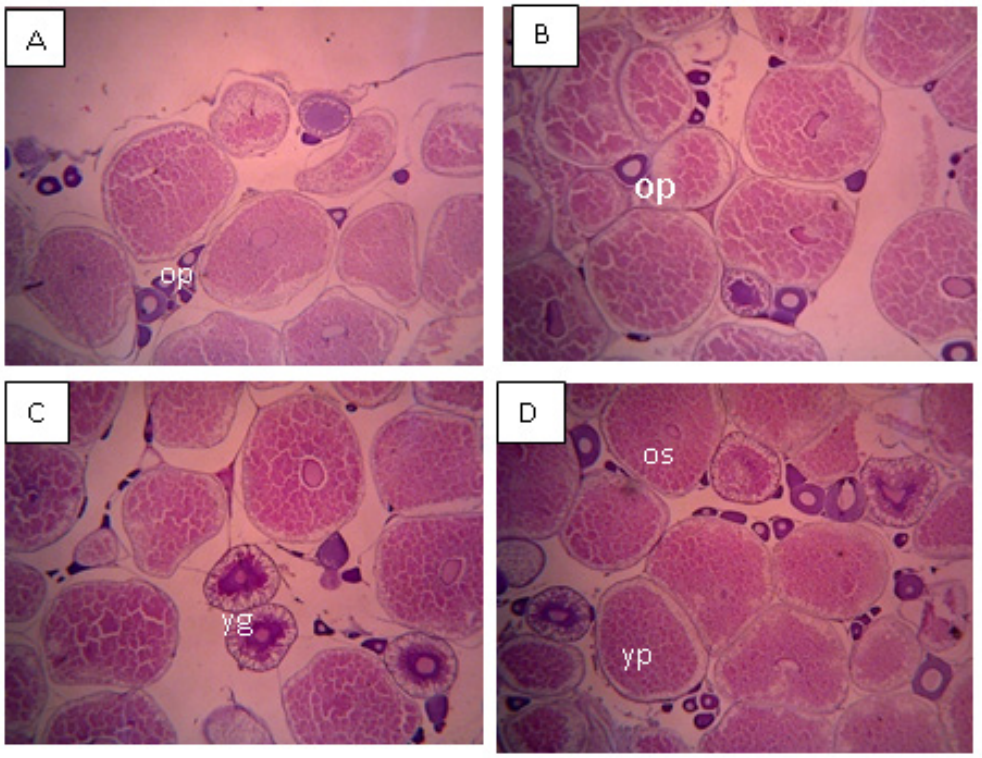

Figure 1. Gonad's maturity of *O. vittatus* at the phase of pre-vitelogenesis and vitelogenesis on 30 days of rearing period; op: primary oocytes; yg:globular yolk, os:secondary oocytes, yp: yolk plate, n: nucleus; A: without AA , B: AA 600 mg/kg fed ration, C: AA 1200 g/kg fed ration, D: AA 1800mg/ kgfed ration.

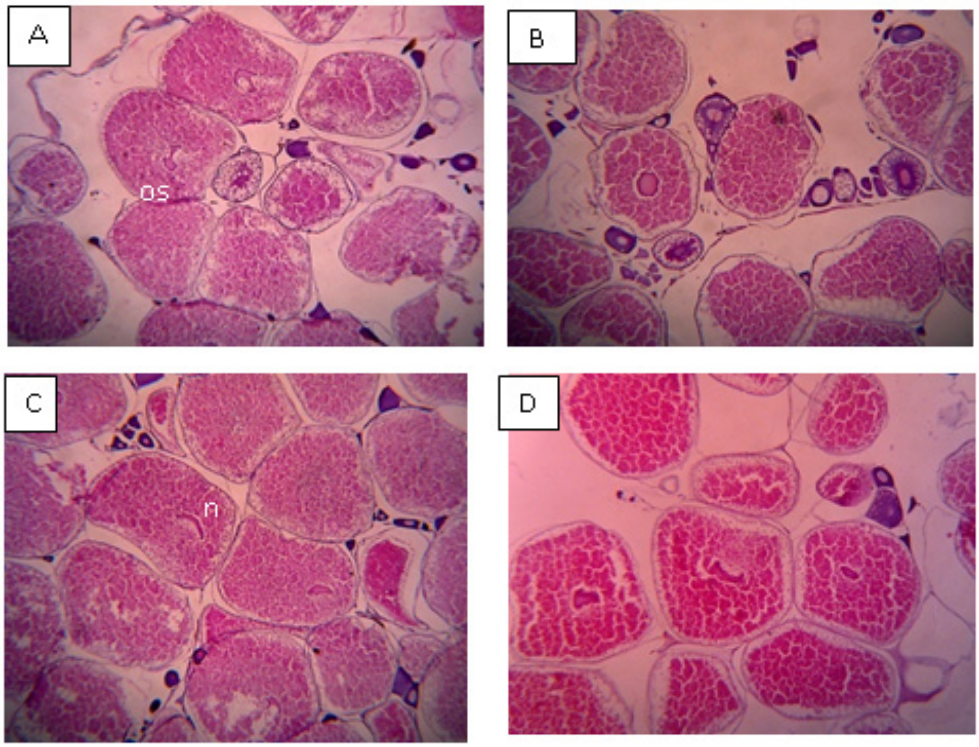

Figure 2. Gonad maturity of *O. vittatus* at vitelogenesis stage performed in 90 rearing-days; op:primary oocytes; os:secondary oocytes, n: nucleus; A: AA absent, B: AA 600 mg/kg fish-fed ration, C: AA 1200 g/kg fish-fed ration, D: AA 1800mg/kg.

rations the presence of secondary oocytes (os) were dominant in numbers though some oocyt primer dan the nucleus of primary oocytes were still obviously present on the periphere. Based on the nucleus position, the ovum were partly ready to enter the stage of gonad maturity i.e.: Germinal Vesicle Break Down (GVBD) but not all ovum were ready to be spawned, though the ovum performance seem like to be ready for it.

Gonad maturity at 90 rearing-days was dominated by the presence of secondary oocytes, especially that of AA supplemented at 1200 mg/kg fish-fed ration and so 1800 mg/kg. The *O. vittatus* ready to enter gonad maturity of Germinal Vesicles Break Down (GVBD) or level IV where nucleus migrated to the periphere and so called as germinal vesicle, from the oocyte periphere to the microphyle (Santos-silva et al., 2015) leads to the position of being ready for spawning. On the other hand, the absence of AA or when AA present at the dosage of 600 mg/kg fish-fed ration, the data showed if the primary oocytes were still present though some ova have already started to enter gonad maturity Level IV. During the development of gonad of the *O. vittatus* there was no atresia found means that all ovum were well develop due to the treatments (supplementation of AA on the fish-ged rations) given to the adult female fish. Sivakumaran reported gonad maturity of carp fish in Victoria, he stated that some of the research's objects had atresia on their ovum caused the ovum failed to develop since there was not enough nutrient available in the nature (Sivakumaran et al., 2003). Another research paper said that in the Lamprey fish, the female gonad contain more ascorbic acid (130-133,8 μg/g) than male ones (79,3-104,1 μg/g), the higher concentration of AA the better quality of eggs would be produced (Moreau & Dabrowski, 1998) it is still unclear from the evolutionary perspective when the ability to synthesize the vitamin first appeared in the animal kingdom and how frequently the trait has been lost. We report here ascorbic acid biosynthesis ability in sea lamprey (Petromyzon marinus). Based on its processes of oocytes development, the *O. vittatus* can be group to the partial spawner fish, i.e.: a type of fish which does not release all eggs simulataneously at once, but only some eggs which related with the oocyt diameter size (Syandri & Azrita, 2015); (Kanta et al., 2009). Current data also noted if the oocyt size was still varied means if these ova were still grown and developed (Hibiya, 1982).

Oocyt diameters

The average size of oocyt diameter at 90 rearing days varied from 0.857 mm when AA was supplemented at 600 mg/kg of the fish-fed ration to the largest of 0.966 mm when AA was supplemented at 1800 mg/kg fish fed ration. These data, however, were not significantly different ($p > 0,05$) (Table 1).

Figure 3 shows the oocyt diameter of each treatment where the diameter increased in every two weeks observation during the rearing period of 90 days. The smallest size of 1.011 mm oocyt diameter was reached when the fish fed did not added with contrastingly when AA was supplemented at 1,800 mg/kg fish fed and reared for 90 days the ovum reached to the size of 1.125 mm.

The oocyt diameter of 90 rearing days of the *O. vittatus* supplemented by AA showed a positive effect where most of the ovum were develop thoroughly in different sizes, along the ovum development this situation led to cause different size of egg diameter. The variation in the ovum diameter was strongly related to spawning process since the *O. vittatus* was a partial spawner and so they do not release all eggs they produce but depends on the oocyt diameter (Sivakumaran et al., 2003). The larger the oocyt the more chance of them to be fertilized and oocyt to be larvae. *O. vittatus* was characterized by variable size of oocyt diameter. Based on this variation in egg sizes, the length period of spawning can be predicted, when all ovum in the ovarium are similar in diameter sizes, the spawning process take shorter time than when the diameter size is the variable. In contrast, when the *O. vittatus* fish spawn in a long time period or even spawn continously, it

Table 1. Relationship between AA supplementation and Oocyt diameter (mm) of *O.vittatus*

Treatments	Observations at 2 week interval						Average
	I	II	III	IV	V	VI	
A	0.700	0.847	0.878	0.925	1.004	1.011	0.894[a]
B	0.613	0.721	0.878	0.899	0.953	1.078	0.857[a]
C	0.848	0.923	0.948	0.944	0.950	1.077	0.948[a]
D	0.824	0.874	0.973	0.988	1.014	1.125	0.966[a]

Values are Means±SD (n = 3) within columns values with different superscripts are significantly different ($p<0.05$).

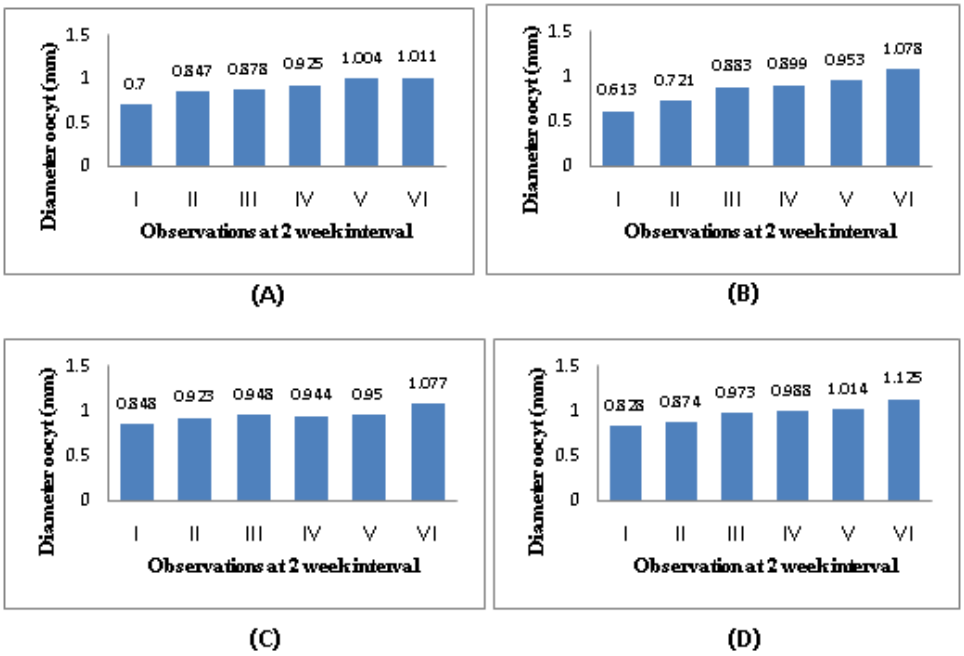

Figure 3. Oocyt Diameter of *O. vittatus* at the supplementation of AA

represents if the eggs in the ovarium are in variable sizes (Sharifuddin & Omar, 2010)·(Abedi et al., 2011). The development process of ovum in the ovarium, is strongly related with length and weight of the fish-body, types and age of the fish. The oocyt diameter of the *Toxotes chatareus* and *Toxotes jaculatri* fish for examples, are vary which related with their fecundity and gonadosomatic index (Simon et al., 2012) spawning season, sex ratio, and fecundity. Spawning season was assessed using monthly changes in gonadosomatic index (GSI).

Larvae survival rate

Following to the artificial spawning, the viability of *O. vittatus* larvae observed at 10 rearing-days and fed with tubifex worm were varied where supplementation of AA at the fish-fed ration of 1,200 mg/kg to the adult female showed the highest larvae viability i.e.: 89.25% but only 20% fish larvae were survived when AA did not supplemented on the fed-ration of the adult female's fish. These data were statistically showed a significant different (p< 0,05) (Figure 4 and Table 2).

Remarks = A: AA is absent in the fish-fed ration; B: Fish-fed ration is supplemented with 600mg/kg AA; C: Fish-fed ration is supplemented with 600mg/kg AA; D: Fish-fed ration is supplemented with 600mg/kg AA

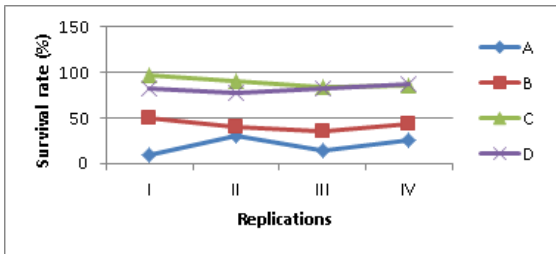

Figure 4. Survival rate (%) of *O.vittatus* after treated with synthetic spawning

Table 2. Survival rate (%) of *O.vittatus* after synthetic spawn treated with synthetic spawning

Treatments	Replications				Average
	I	II	III	IV	
A	10	30	15	25	20^{a}
B	50	40	35	43	42^{b}
C	97	90	84	86	89.25^{cd}
D	82	78	83	88	82.75^{dc}

Values are Means±SD (n = 3) within columns values with different superscripts are significantly different (p<0.05).

Apart from that, the larvae were able to utilize the tubifex worms, to run their own metabolism activities and grow further. Syandri (2013) stated if the *O. vittatus* larvae fed by the tubifex worms resulted in a better growth as well as their

viability, becuase the tubifex worms contain a complete nutritions like water (mg/100 mg fresh weight) 87,19 ± 0,83, rough protein 57 ± 0,58, lipid 13,3 ± 0,06, ash 3,60 ± 0,16 and amino acids 7,18 mg/100 mg dry weight namely: lysine, leusin, arginin, valin, treonin, fenilalanin, isoleusin, tirosin, histidin, methionin, and well digested (Syandri & Azrita, 2015). Muchlisin et al., (2016) has note that survival rate of *Tor Tambra* larvae was increased by papain enzyme supplementation of 27.50 mg / kg and protein 30% because the enzymes able to optimaize protein digestion.

Our result proved that ascorbic acid supplementation accelerated gonad maturity which might leads to increase spawning frequency of the *O vittatus* become more than twice per annuum and result in the increase of larvae production. This will provide high benefit for fish famers.

CONCLUSIONS

Supplementation of AA at the dosage of 1200 mg/kg fish-fed ration could accelerate gonads maturity, development of oocyt diameter, as well as the viability of *O. vittatus* larvae at 90 rearing days. Rematuration was also run in a faster period than other treatment groups.

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