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Physical Characteristics of Java Barb (*Puntius javanicus)* Sperm after Stratification with Percoll Density Gradient Centrifugation

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ABSTRACT: The purpose of this research was to know the physical characteristics of Java Barb sperm which has been separated through sexing with Percoll density gradient centrifugation. Method used was descriptive method. Sperm from male fish which was more than a year old, acquired by pushing gently the stomach to its urogenital opening, immediately brought to the laboratory. Percoll medium concentrations utilized in this research were in 6 levels, they were 10%, 20%, 30%, 40%, 50%, and 60%. Centrifugation was conducted for 5 minutes at 1600 rpm of velocity. Each characteristics observation includes concentration, viability and size of the sperm was done 15 times and then was analyzed with paired T-test. The results showed that there were significant characteristics differences between both formed layers. Top layer of sperm concentration which is supposed to be y sperm was 1.33 more than the bottom layer which is supposed to be x sperm. But for the viability, percentage value of bottom layer sperm was 10% more than the top layer. And the sexing of x and y sperm was supported by the total length gap between top layer sperm (34.19 $\pm$ 1.96 µm) and the bottom layer (36.70 $\pm$ 1.74 µm).
Key words: Male Gamete Cells, Centrifugation, Concentration, Viability, Total Length

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INTRODUCTION

Java barb (*Puntius javanicus*) which is classified on *Puntius* species is one of important freshwater commodities in Indonesia [1] which is originated from a river in Java Island called Brantas River [2]. Offspring sex determining role in eukaryotic organisms is hold by male parent fish with its haploid gamete cells, spermatozoa x and y. Basically, if spermatozoa y is fertilized into the fish egg then the offspring produced will be male and vice versa [3].

Sexing or sperm stratification is a way performed to get different proportion of its natural condition which is 50%:50% [4]. Percoll Density Gradient Centrifugation (SGDP) is a common method used to separate sperm as the procedure is relatively easy to perform and the operation cost is not very expensive [5]. Basic principle of this method is the physical treatment in form of centrifugation to Percoll medium with leveled concentration which causes sperm stratification due to mass and size differences between spermatozoa X and Y [6].

The purpose of this research was to know the physical characteristics of Java Barb sperm which has been separated with Percoll density gradient centrifugation, and the result was expected to be a reference in fishery improvement.

MATERIAL AND METHODS

Male Java barb (*P. javanicus*) used was more than one year old and acquired from a fish farmer in Pare, Kediri. Fish sperm was acquired by pushing gently the stomach to its urogenital opening and then immediately brought to the laboratory. Method used in the research was descriptive method. The layers of sperm formed by centrifugation were observed for 15 times and to determine the differences among the layers, paired t-test was used [7].

Percoll medium concentrations used in the research were in six levels, they were 10, 20, 30, 40, 50, and 60%. The medium was prepared in 15 ml centrifuge tube consecutively from the highest density to the lowest one, each of them was 1 ml. One milliliter of sperm was added on the Percoll medium arrangement and was centrifuged for 5 minutes at 1600 rpm velocity. Sperm layers which were successfully separated, were taken and prepared in a centrifuge tube filled with 3 ml of Tris (hydroxymethyl) aminomethane egg yolk and then re-suspended by 960 rpm centrifugation for 5 minutes.

Observation on sperm concentration was a modification of the research of Fauvel et al. [8] which was done by mixing 0.5 ml sperm with some diluent consisting of 50 ml distilled water, 2 ml eosin and 1.5 g NaCl to reach

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the limit at 1.01 of Thoma pipette. Both were homogenized and 3-4 drops were disposed of. Then, they were prepared in haemocytometer chamber and observed under the light microscope with 400 times magnification. The formula for sperm concentration count is as follows:

N = squares count x squares volume x dilution factor

Measurement of live spermatozoa was conducted on sperm drop on the objecting glass using eosin staining. Both substances were prepared as smears, using other objecting glass which formed 45^0 angle. Smears were observed under a microscope with 1000 times magnification. Spermatozoa viability count, based on the comparison between counted spermatozoa which did not absorb the red color and the total spermatozoa count and stated in percentages [9].

Information on the shape and size of the sperm, of the head or the tail are important aspects of organism's spermatozoa characteristic presentation [10]. So, to acquire that information, microscopic observation on the sperm is needed. The specimen used to observe live sperm percentage can also be used for morphological observation [11]. But this observation was only focused to measure the length and the diameter of the head, the length of the tail and the total length of the sperm.

RESULTS

After performing the stratification using Percoll density gradient centrifugation method, average sperm concentration formed in two layers was presented in Table 1. Based on the paired t-test calculation, there were significant differences between sperm concentration in the top layer and the bottom one. Sperm accumulated in the top layer ($42.35 \pm 7.09 \times 10^6$ cells/ml) had more concentration by 1.33 times compared to the sperm in bottom layer ($31.84 \pm 4.94 \times 10^6$ cells/ml).

Average sperm viability percentage of Java barb after stratification using Percoll density gradient centrifugation method was presented in Table 2. Average sperm viability in top and bottom layer after stratification also showed very significant differences. Percentage of live spermatozoa in the bottom layer was bigger than the top layer by ± 10%.

Average measurement of length and diameter of the head, the length of the tail and the total length of the sperm were presented in Table 3. Based on the Java barb sperm measurement after stratification, sperm in the bottom layer had longer total length compared to the sperm in the top layer by 7.06%.

Table 1. Java barb sperm concentration

Category	Average ± St.dev
Top layer	$42.35 \pm 7.09 \times 10^6$ cells /ml
Bottom layer	$31.84 \pm 4.94 \times 10^6$ cells /ml

Table 2. Java barb sperm viability percentage

Category	**Average ± St.dev**
Top layer	67.83 ± 8.92%
Bottom layer	75.29 ± 8.8%

Table 3. Java barb sperm measurement

Parameters	**Sperm**	
	Top layer	**Bottom layer**
Head length	2.05 ± 0.17 µm	3.67 ± 0.42 µm
Head diameter	1.73 ± 0.9 µm	2.79 ± 0.28 µm
Tail length	32.14 ± 1.83 µm	33.03 ± 1.34 µm
Total sperm length	34.19 ± 1.96 µm	36.70 ± 1.74 µm

DISCUSSION

Velocity and the centrifugation duration were the reason for different concentration between both sperm layers. Low sperm sediment in the bottom layer during this research was caused by centripetal force effect was bigger than the centrifugal force [12]. Centripetal force kept the sperm away from the tube center or only limited number reached the tube base. Therefore, most sperm diffused in the top layer [13]. Based on that information,

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centrifugation at 1600rpm velocity for 5 minutes on Java barb sperm resulted on more sperm swarming in the top layer.

Naturally, sperm produced by male parent fish was live sperm with high motility, live sperm with low motility or static and dead sperm [14]. Sperm with low motility or dead sperm were supposed to be in the top layer. During the centrifugation process, sperm with good quality will swim over Percoll medium gradient. Whereas sperm with low motility was only carried by the centripetal force, could not swim over the Percoll medium gradient, stayed in the low gradient. Dead sperm mass was supposed to be lessened due to hard bumps, the membrane was so damaged that its intercellular material gushed out and head separated from the tail that it floated on the tube surface [15]. For that reason, more than 30% of sperm in top layer counted as dead sperm.

Henkel and Schill [16] stated that centrifugation at the process of stratification cause sperm carried by percoll medium far away from central of tube. Besides friction among sperm, percoll medium composed of silica colloidal sized 15-30 nm is also experienced the force of attraction to maintain a gradient during the centrifugation process. Percoll is known could cling into biological material membrane. It may allow lipid and protein of sperm membrane damaged so permeability disturbed. Then Mantayborbir et al. [17] supported that presentation of died spermatozoa could increased when electron density mitochondrial matrix has decreased because of plasma membrane infrastructure changes. In such a condition dyestuff would be easy to pass through a membrane spermatozoa. Whereas in alive sperm, semi permeable characteristic of membrane is could pumping so dyes with Eosin-Nigrosin not able to pass through.

Java barb sperm measurement results supported the researchers supposition on the ability of Percoll density gradient centrifugation method to collect y sperm in the top layer and x sperm in the bottom layer fit to their characteristics. The fundamental of SGDP method itself is the ability of sperm to swim over Percoll medium gradient [15]. According to Carvalho et al. [18] x spermatozoa has bigger size than y spermatozoa by $\pm$ 6%. Then Yuwono [19] added that the sedimentary ability is more likely possessed by bigger size molecules due to centripetal force effect during centrifugation. Therefore, x sperm could swim over Percoll medium gradient and reached the tube base.

CONCLUSION

Java barb sperm stratification using Percoll density gradient centrifugation method was considered successful with some indicators such as the formation of top and bottom layers and the significant differences between them. Concentration, viability and total length of sperm in the top layer which was supposed to be y sperm was (42.35 $\pm$ 7.09 × 10^6 cells/ml); (67.83 $\pm$ 8.92%); (34.19 $\pm$ 1.96 µm). While concentration, viability and total length of sperm in the top layer which was supposed to be x sperm was (31.84 $\pm$ 4.94 × 10^6 cells/ml); (75.29 $\pm$ 8.8%); (36.70 $\pm$ 1.74 µm).

Recommendation

For future researches, we suggest stratification of java barb sperm to be researched with its other physical characteristics such as its motility, abnormality and capable of fertilizing eggs. In addition, it need to be conducted stratification of java barb sperm using another methods, beside percoll density gradient centrifugation.

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