



# Effect of egg yolk on the spermatozoa quality of the botia *Chromobotia macracanthus* (Bleeker, 1852) (Cyprinidae) after short-term cryopreservation

<sup>1</sup>Abinawanto, <sup>1</sup>Rinda Wulandari, <sup>2</sup>Zainal A. Muchlisin

<sup>1</sup> Biology Department, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok, West Java, Indonesia; <sup>2</sup> Department of Aquaculture, Faculty of Marine and Fisheries, Syah Kuala University, Banda Aceh, Indonesia. Corresponding author: Abinawanto, abinawanto.ms@sci.ui.ac.id

**Abstract.** The egg yolk effect combined with 10% of methanol to preserve botia, *Chromobotia macracanthus* spermatozoa for 24 hours in liquid nitrogen has been studied. The concentration of egg yolk used in this study were, 0%, 5%, 7%, 9%, 11%, 13%, 15%, and 17%, respectively. As the positive control, the synthetic cryoprotectant carboxy methyl cellulose (CMC) was used. The sperms were diluted with the combination of 10% methanol, glucose extender and egg yolk (1 part of sample + 4 parts of solvent) and were equilibrated for 2 hours at 4°C before frozen for 24 hours in liquid nitrogen. After frozen, sample (sperms) were thawed for 13 sec at 40°C. Spermatozoa viability, motility, and abnormality, were then observed. Ten percent of methanol combined with 15% of egg yolk could preserve spermatozoa which maintain either sperm motility (96.43±1.49%) or sperm viability (84.25±1.26%) and reduced the abnormality up to 11.50±1.29%, respectively. Besides, the combination of 15% of egg yolk and 10% of methanol showed the highest fertility rate (50.64±4.37%).

**Key Words:** *Chromobotia macracanthus*, egg yolk, spermatozoa preservation, fertility rate.

**Introduction.** Botia fish (*Chromobotia macracanthus* (Bleeker, 1852)) is one of the Indonesian fresh water endemic species, originated from Sumatera and Borneo (Kottelat et al 1993). This is the most popular freshwater fish for aquarium in Indonesia, just after Asian arowana, *Schleropages formosus* (Satyani et al 2006). In the local market in Indonesia the price reach about IDR 200,000 per individual. Several studies in Indonesia on the botia morphology have been reported by Sudarto & Pouyaud (2006), and Dahruddin (2011). While, studies on egg production of botia in Indonesia has been reported by Legendre et al (2012). Further, Dey et al (2015) has studied spawning biology, embryonic development, and captive breeding of botia Dario in India. Based on IUCN Red List, *C. macracanthus* is threatened by over fishing and habitat perturbation (Sudarto & Pouyaud 2006). Therefore, cryopreservation is crucially needed to prevent the species extinction. Sperm cryopreservation is one of the methods to solve this problem (Muchlisin et al 2004; Muchlisin & Siti-Azizah 2009; Chew & Zulkafli 2012; Muchlisin et al 2015).

The cryopreservation is the method to keep the genetic materials such as, oocyte, spermatozoa and embryo, against the extreme conditions such as low temperature in a certain period of time (Stacey & Day 2007; Abinawanto et al 2011; Tsai & Lin 2012). Several factors are influencing the success of the cryopreservation, such as cryoprotectants, extender, anti-oxidant and freezing and thawing. The combination of cryoprotectant, extender, and methanol have been reported to preserve fish spermatozoa, such as *Mystus nemurus* (Muchlisin et al 2004; Muchlisin & Siti-Azizah 2009), *Clarias gariepinus* (Muchlisin et al 2010; Muchlisin et al 2015), *Osphronemus goramy* (Abinawanto et al 2012a, 2017), *Barbonymus gonionotus* (Abinawanto et al 2013, 2016), salmonid, *Salmo gairdneri* spermatozoa (Harvey & Ashwood-Smith 1982),

common carp *Cyprinus carpio* (Withler 1982; Harvey 1983; Horvath et al 2003), and rainbow trout, *Salmo gairdneri* (Stoss & Donaldson 1983). The effect of honey and 10% of methanol on *O. goramy* spermatozoa after short-term storage in -34°C has been reported by Abinawanto et al (2017). In addition, the skim milk was suitable as the cryoprotectant for preserving *B. gonionotus* spermatozoa (Abinawanto et al 2016). However, the quality of *C. macranchanthus* spermatozoa after storage in liquid nitrogen has not been reported, yet. Therefore, the present study was to evaluate the effect of egg yolk combined with 10% of methanol on spermatozoa quality of *C. macranchanthus* spermatozoa after freezed in liquid nitrogen for 24 hours.

## Material and Method

**Time and site.** The study was conducted in March-October 2017, at the Genetics Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province, Indonesia.

**Broodfish.** A total of 40 mature males of botia, *C. macranchanthus* were obtained from a Research Institute for Ornamental Fish Culture, Depok, West Java, Indonesia, and the fish samples were transported to the indoor Aquatic Biology Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. The broodfish were acclimatized in laboratory conditions and fed a commercial diet for 14 days in the 45 cm x 30 cm x 30 cm plastic tank. After acclimatization, each of plastic tanks were stocked with four fishes with an average initial weight of 60.47±10.34 g. The total of nine plastic tanks were equipped with continuous aeration and a black plastic mesh lid to minimize disturbances and prevent fish from jumping out. Nine experimental groups were assigned to four times replication in a completely randomized design.

**Preparation of the extender glucose solution.** Extender glucose solution was prepared based on Jodun et al (2007). The stock of extender glucose solution was prepared by dissolving of the 5.4 g C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> and 0.17 g KCl with distilled water (DW) up to 100 mL. The extender glucose solution was then kept at 4°C prior to use for experiment.

**Preparation of the activator and the 0.5% eosin-Y solutions.** The activator solution was prepared by diluting 45 mM NaCl, 5 mM KCl, and 30 mM Tris with DW up to 100 mL (Perchec et al 1995). The 0.5% of eosin-Y solution was prepared by diluting 0.5 g of the eosin-Y with DW up to 100 mL (WHO 1988).

**Semen/sperm collection and dilution.** The sperm was collected by hand stripping method and was put in 1.5 mL of cryotube, according to Sunarma et al (2007). The ratio among the ejaculated semen/sperm and the solvent was 1:4 according to Jodun et al (2007). The sperm was added to the solvent solution in the cryotube. The composition of each component of the solvent solution and the ejaculated sperm is presented in Table 1.

Table 1

Composition of sperm, methanol, extender glucose solution and egg yolk according to experimental group

Composition	Experimental group								
	C1	C2	EY 5%	EY 7%	EY 9%	EY 11%	EY 13%	EY 15%	EY 17%
Semen/sperm (µL)	500	500	500	500	500	500	500	500	500
10% methanol (µL)	500	500	500	500	500	500	500	500	500
Extender glucose solution (µL)	4449	4500	4220	4110	4000	3895	3785	3675	3565
Egg yolk/EY (µL)	0	0	280	390	500	605	715	825	935
CMC 1% (µL)	51	0	0	0	0	0	0	0	0

C1 = 1% CMC (carboxy methyl cellulose), without egg yolk (0% egg yolk); C2 = 0% egg yolk (without egg yolk); EY = egg yolk.

**Sperm equilibration, freezing and thawing.** The diluted sperm was then equilibrated at 4°C for 2 hours according to modified method by Lahnsteiner et al (2002). The diluted sperm was freezing in liquid nitrogen for 24 h based on Abinawanto et al (2011). The frozen sperm was incubated at 40°C for 13 sec prior to analysis of the quality based on Jodun et al (2007).

**Sperm quality analysis.** Fresh sperm color was observed by visual, whereas sperm volume was measured by the cryotube with scale. Fresh sperm pH was measured by standardized pH paper (pH range 5-10). The parameter of the spermatozoa analyzed were spermatozoa motility, viability, and abnormality rate, respectively. All of the spermatozoa analyses parameters were observed under trinocular microscope (Boeco) equipped with the digital eye piece camera (MDCE-5a). This microscope was connected to the computer equipped by the image driving software (Scopephoto 2.0.4). Spermatozoa motility was analyzed by subjective method based on Rurangwa et al (2004), whereas spermatozoa viability was analyzed based on Salisbury et al (1978).

**Egg collection and artificial fertilization.** Egg collection and artificial fertilization were conducted based on Legendre et al (2012). Fertilized egg and unfertilized egg were counted. The unfertilized eggs were identified by their opacity (Muchlisin et al 2015). Fertilization rate was estimated by using the following formula:

$$\text{Fertilization rate (\%)} = \frac{\text{fertilized eggs}}{\text{total number of eggs in sample}} \times 100$$

**Statistical analysis.** All data were analyzed by one-way ANOVA test and followed by Tukey's multiple comparison test (Zar 1974) using the SPSS software version 13 for Windows. All probability values were set at 0.05 level of significance.

**Results.** Fresh sperm are milky white, pH 7.9±0.0, and 0.63±0.24 mL of volume per ejaculate (Table 2).

Table 2

Fresh semen/sperm evaluation and spermatozoa analyses of *C. macranchanthus* spermatozoa, before freezing

Color	Volume (mL)	pH	Motility (%)	Viability (%)	Abnormality (%)	Fertility (%)
Milky white	0.63±0.24	7.9±0.0	91.70±6.67	83.25±5.91	16.00±3.46	80.89±7.46

Value are means±SD of four replicates

The viable sperm showed green color on the sperm head (Figure 1a), while the non-viable sperm showed pink or red color on the sperm head (Figure 1b). Microcephalus (abnormal head spermatozoa) was found in this study (Figure 2). Based on observation in this study, the fertilized eggs were transparent and unfertilized ones were opaque and white (Figure 3).

Based on one way ANOVA test, there were significant effect ( $p < 0.05$ ) of various concentration of egg yolk on sperm motility, viability, abnormality, and fertilization rate, respectively, compared to control-2, C2 (10% methanol; 0% egg yolk) (Table 3). According to the Tukey multiple comparison test, the concentration of 15% of egg yolk showed the highest post-thaw sperm motility (96.43±1.49%), viability (84.25±1.26%), and fertilization rate (50.64±4.37%), but, the lowest post-thaw abnormality (11.50±1.29%).

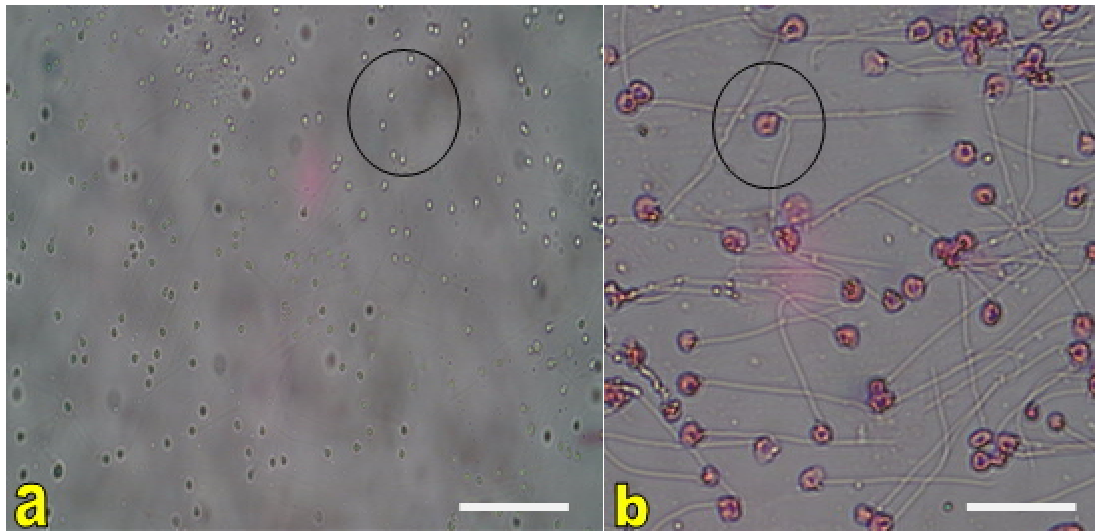


Figure 1. Viable spermatozoa (A) [10x40]; non-viable spermatozoa (B) [10x100]; Bar = 10  $\mu$ m.

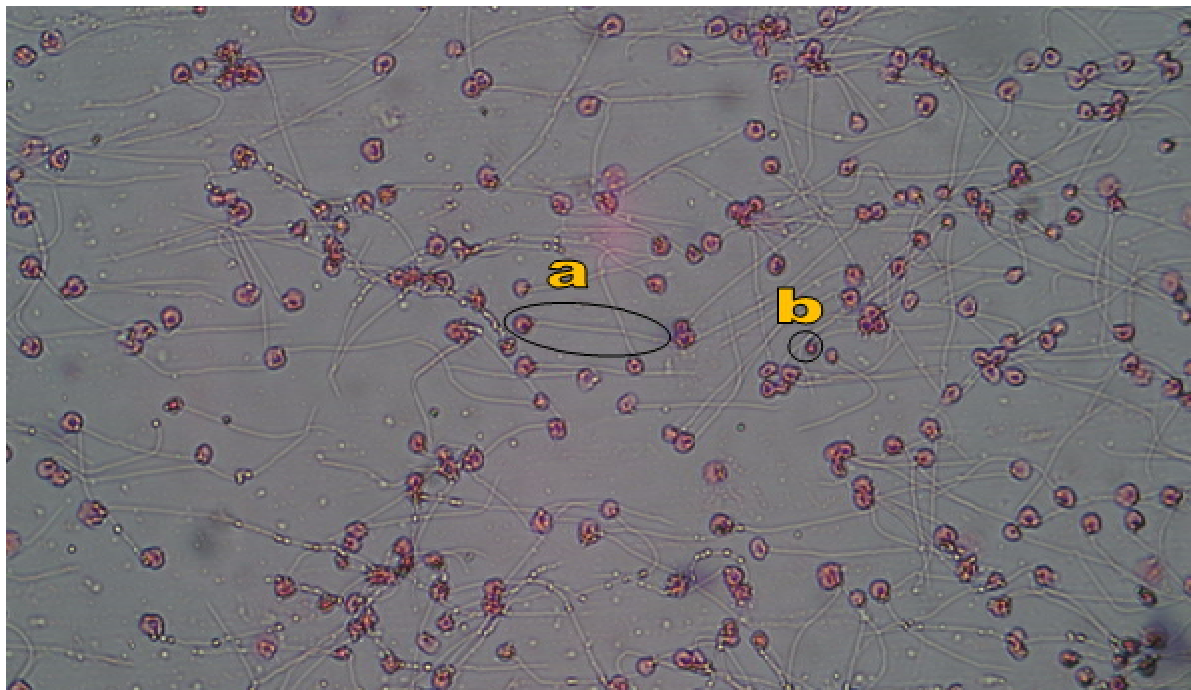


Figure 2. Normal spermatozoa (A); Microcephalus spermatozoa (B); 10x100; Bar = 10  $\mu$ m.

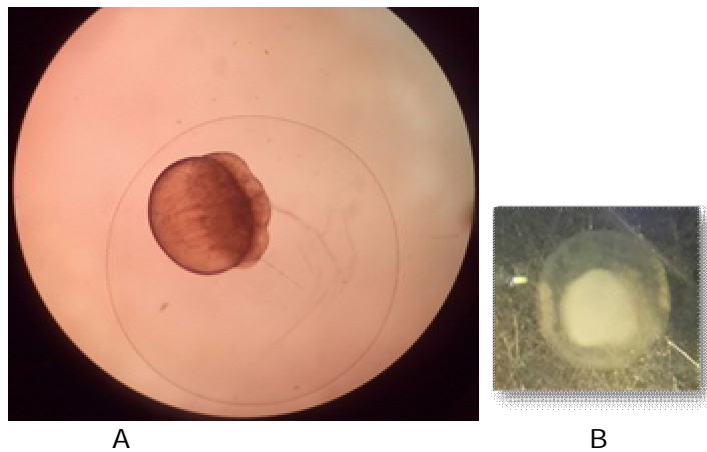


Figure 3. Fertilized egg (A); unfertilized egg (B); 10x100; Bar = 10  $\mu$ m.

Table 3

Viability, abnormality, and motility of *C. macranchanthus* spermatozoa, 24 hours after freezing

Composition	Experimental group								
	C1	C2	EY 5%	EY 7%	EY 9%	EY 11%	EY 13%	EY 15%	EY 17%
Motility rate (%)	82.15±1.57 <sup>b</sup>	64.93±3.92 <sup>a</sup>	79.46±3.48 <sup>b</sup>	90.48±3.45 <sup>c</sup>	92.51±1.77 <sup>cd</sup>	92.25±1.70 <sup>cd</sup>	95.57±1.78 <sup>cd</sup>	96.43±1.49 <sup>d</sup>	92.61±1.21 <sup>cd</sup>
Viability rate (%)	78.25±4.03 <sup>ab</sup>	70.75±5.38 <sup>a</sup>	75.50±2.65 <sup>ab</sup>	74.00±3.56 <sup>ab</sup>	78.75±6.65 <sup>ab</sup>	80.50±7.51 <sup>ab</sup>	72.00±2.45 <sup>a</sup>	84.25±1.26 <sup>b</sup>	77.50±7.19 <sup>ab</sup>
Abnormality rate (%)	15.25±2.87 <sup>ab</sup>	19.75±5.50 <sup>b</sup>	18.50±2.38 <sup>ab</sup>	20.25±3.86 <sup>b</sup>	18.00±3.65 <sup>ab</sup>	18.50±2.38 <sup>ab</sup>	12.75±1.71 <sup>ab</sup>	11.50±1.29 <sup>a</sup>	15.25±3.10 <sup>ab</sup>
Fertilization rate (%)	39.50±5.15 <sup>abc</sup>	31.78±1.36 <sup>a</sup>	40.60±5.28 <sup>abc</sup>	36.52±8.06 <sup>a</sup>	39.63±5.94 <sup>abc</sup>	49.60±4.93 <sup>bc</sup>	43.33±3.93 <sup>abc</sup>	50.64±4.37 <sup>c</sup>	38.17±5.15 <sup>ab</sup>

C1 = 1% CMC, without egg yolk (0% egg yolk); C2 = 0% egg yolk (without egg yolk); EY = egg yolk. Values are means±SD of four replicates. Mean values having the same superscript are not significantly different ( $p > 0.05$ ).

**Discussion.** The finding revealed that the effect of 15% of egg yolk + 10% methanol + glucose-base extender on the percentage of spermatozoa motility 24 hours after freezing was higher (96.43±1.49%) than those observed in other fish species such as *O. goramy* (80.48±7.18%; Abinawanto et al 2017), *B. gonionotus* (83.23±3.27%; Abinawanto et al 2016), *O. goramy* (75.95±4.76%; Abinawanto et al 2015), *Mystus nemurus* (58%±2.8%; Muchlisin et al 2004), *Brachydanio rerio* (51%; Harvey et al 1982), *Oreochromis mossambicus* (70%; Harvey 1983), tilapia's fish (40-80%; Chao et al 1987), *C. carpio* (55%; Akcay et al 2004), *Lota lota* (46.6±8%; Lahnsteiner et al 2002) and *Osteochilus hasseltii* (63.33%; Sunarma et al 2007).

Cryoprotectants and extenders are the crucial factors that cause the spermatozoa quality after sub-zero freezing, such as 5% of methanol + 15% of skim milk (Stoss & Donaldson 1983; Chao et al 1987); 5% of glycerol + fish ringer (Abinawanto et al 2015), 13% of DMSO + 189 M extender (Abinawanto et al 2011); 10% of methanol + 15% of egg yolk (Abinawanto et al 2012a); 6% of glucose + 10% of methanol (Abinawanto et al 2009), 10% of methanol + glucose-base extender + 15% of egg yolk (Abinawanto et al 2013), and 15% of DMSO (Horton & Ott 1976; Sunarma et al 2007). The combination of 15% of egg yolk + 10% methanol + glucose-base extender has also maintained the viability rate (84.25±1.26%). This result (viability rate) was higher than our previous study (80.48±7.18%; Abinawanto et al 2017). Besides, the viability was also higher compared to other species like *B. gonionotus* (81.75±8.22%; Abinawanto et al 2016), *C. carpio* (20%; Withler 1982; 58%; Horton & Ott 1976), *O. goramy* (75.5±5.43%; Abinawanto et al 2015), *O. goramy* (63.5%; Abinawanto et al 2011), and *B. gonionotus* (77.25%; Abinawanto et al 2009). In addition, the combination of 15% egg yolk + 10% methanol + glucose-base extender has also maintained the percentage of abnormality rate (11.50±1.29%). The abnormality rate in this study (11.50%) was lower compared to our previous study using 0.7% of honey solution + 10% of DMSO (28.25±1.5%; Abinawanto et al 2017), 5% of glycerol + fish ringer (14.83±2.79%; Abinawanto et al 2015), 6% of glucose + 10% of methanol (45%; Abinawanto et al 2009), and also lower than other species like *B. gonionotus* (26.25±1.89%; Abinawanto et al 2016), *O. goramy* (29%; Abinawanto et al 2011) and *M. nemurus* (62.65-83.38%, Muchlisin & Siti-Azizah 2009).

Fertilization rate after freezing in this study (50.64±4.37%) was lower than those reported in other species such as, *Lota lota* (78.1±2.7%; Lahnsteiner et al 2002) and *C. gariepinus* (80.67±5.69%; Muchlisin et al 2015). Thawing procedures at 40°C for 13 seconds was effective for 2 mL of cryogenic tubes. Forty Celcius degree was chosen in this study, because this is easy to achieve using heating devices in our temperature conditions. Methanol as an intracellular cryoprotectant significantly improved motility of cryopreserved sperm. Methanol was employed as successful intracellular cryoprotectant in *B. rerio* (Harvey et al 1982); *O. mossambicus* (Harvey 1983); tilapia's fish (Chao et al 1987); *C. carpio* (Horváth et al 2003), *M. nemurus* (Muchlisin & Siti-Azizah 2009), *O. goramy* (Abinawanto et al 2011, 2012a, 2012b), *B. gonionotus* (Abinawanto et al 2009, 2013, 2016). Besides, in this study was applied egg yolk as an extracellular

cryoprotectant. Egg yolk was also successful as an extracellular cryoprotectant as reported in other species, such as in *B. gonionotus* (Abinawanto et al 2013), and *C. gariepinus* (Muchlisin et al 2015). Utilization of other natural cryoprotectant and natural extender, for example skim milk, honey, sucrose, glucose, sugarcane water, soybean solution and coconut water were also reported (Muchlisin 2005; Muchlisin et al 2010, 2015; Abinawanto et al 2016, 2017; Abinawanto & Putri 2017). According to Muchlisin (2005), natural cryoprotectant is less or non-toxic and environment-friendly cryoprotectant, so utilization of natural cryoprotectant as alternative is a promising.

**Conclusions.** The combination of 10% of methanol and 15% of egg yolk is an effective concentration and is recommended to be used as a cryoprotectant for sperm cryopreservation of *Chromobotia macracanthus* species.

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Authors:

Abinawanto, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, West Java, Indonesia, e-mail: abinawanto.ms@sci.ui.ac.id

Rinda Wulandari, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, West Java, Indonesia, e-mail: rindawulandari20@gmail.com

Zainal A. Muchlisin, Department of Aquaculture, Faculty of Marine and Fisheries, Syah Kuala University, Banda Aceh, Indonesia, e-mail: muchlisinza@unsyah.ac.id

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