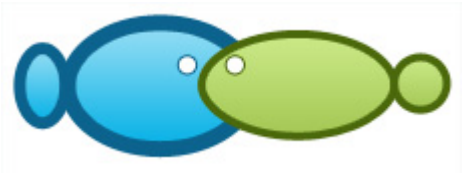

Sperm motility of giant gourami (*Osphronemus goramy,* Lacepede, 1801) at several concentrations of honey combined with DMSO after short-term storage

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Abstract. The effect of the commercial honey combined with 10% of Dimetyl Sulfoxide (DMSO) to preserve spermatozoa of gourami, *Osphronemus goramy* for 48 hours at -34°C has been studied. The concentrations of honey used in this study were 0%, 0.1%, 0.3%, 0.5%, 0.7%, and 0.9% (v/v), respectively. The sperms were diluted with the combination of 10% of DMSO, fish ringer and honey (1 part of sample + 3 parts of solvent) and were equilibrated for 10 minutes at 4°C before frozen for 48 hours at -34°C. After freezing, sperms were thawed for 1-2 min at 40°C. Spermatozoa motility was then observed. The ANOVA test showed that honey had a significant effect on the motility rate ($p < 0.05$). The Tukey multiple-range test revealed that 10% of DMSO combined with 0.7% of honey gave the highest motility (80.48±7.18%). Therefore, it is concluded that 0.7% of honey is the suitable concentration for gourami spermatozoa.
Key Words: *Osphronemus goramy*, commercial honey, spermatozoa motility.

Introduction. Gourami, *Osphronemus goramy* is one of the indigenous freshwater fish species in Indonesia which has high meat quality, thus accounting for its commercial importance (Sitanggang & Sarwono 2006). Gourami production was hindered by limitation of the high quality of broodstock (Sunarya 2007). Therefore, sperm cryopreservation is needed to solve the problem related to the broodstock supply especially for male broodstock. In order to get the highest quality and quantity of spermatozoa, sperm should be collected during spawning seasons (Muchlisin et al 2004). Cryopreservation of gourami spermatozoa using sucrose, skim milk and egg yolk as cryoprotectants has been reported by Abinawanto et al (2011), Abinawanto et al (2012a), and Abinawanto et al (2012b). However, the efficacy of honey and DMSO on gourami spermatozoa has never been evaluated.

According to Chao & Liao (2001) cryoprotectants are needed to avoid the spermatozoa from the cold shock and hot shock effect. However, cryoprotectants cause toxicity of cellular systems (Muchlisin & Siti-Azizah 2009). According to Tsai & Lin (2009), cryoprotectant toxicity depends on type, concentration, temperature, and exposure period. To avoid the toxicity of cryoprotectant finding the natural less or nontoxic and environment-friendly cryoprotectant as the alternative (Muchlisin et al 2005) is a must.

According to Anil et al (2011), a non toxic cryoprotectant is important for cryopreservation. Utilization of cryoprotectant and extender at various concentrations were reported by several researchers, for example the combination of skim milk and 5% of methanol was used for preserving Java barb, *Barbonymus gonionotus* spermatozoa after 24 hours freezing (Abinawanto et al 2016). Besides, cryopreservation of African catfish, *Clarias gariepinus* spermatozoa using egg yolk, glucose, honey and coconut water was reported by Muchlisin et al (2015). In the previous study, Abinawanto et al (2013) applied the combination of egg yolk and 5% of methanol to preserve Java barb spermatozoa after 24 hours freezing. Skim milk at concentration of 20% has been

reported to be suitable for cryopreservation of Java barb (Abinawanto et al 2016) and gourami (Abinawanto et al 2012a; Abinawanto et al 2012b) spermatozoa, respectively. Besides, glucose has been applied also as a cryoprotectant for cryopreservation of African catfish (Muchlisin et al 2015) and Java barb (Abinawanto et al 2009) spermatozoa. Meanwhile, sucrose has been used as a cryoprotectant for cryopreservation of gourami spermatozoa (Abinawanto et al 2012a). Further, Muchlisin et al (2015) reported the role of honey as a cryoprotectant for cryopreservation of African catfish spermatozoa. However, the commercial honey as the potential cryoprotectant has never been tested for cryopreservation of gourami spermatozoa.

Therefore, the objective of the present study was to find the best cryoprotectants and their concentrations for short-term cryopreservation of the gourami spermatozoa. Herein, we evaluated the efficacy of the natural cryoprotectant (commercial honey) at different concentrations.

Material and Method. The study was conducted for six months between November 2009 to April 2010, at the Genetics Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Provinces, Indonesia.

Preparation of broodfish. A total of 24 mature broodstock of gourami males were obtained from a private commercial hatchery in Parung, Bogor, West Java, Indonesia, and the broodstocks were transported to the Indoor Aquatic Biology laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. The fishes were acclimatized in laboratory conditions and maintained with a commercial diet for 14 days in the 2000-L square concrete cement tank. The fish pond was equipped with closed re-circulation system and a black plastic mesh lid to minimize disturbances and prevent fish from jumping out. Fishes were fed the commercial diet and leaf of *Alocasia macrorrhiza* two times daily *ad libitum* at 08.00 AM and 17.00 PM.

Preparation of Ringer solution. The Ringer solution was prepared according to proposed method by Draper et al (2008). A stock of Ringer solution was prepared by dissolving 3.25 g NaCl; 0.125 g KCl; 0.175 g $CaCl_2.2H_2O$; and 0.1 g $NaHCO_3$ with distilled water up to 500 mL and then kept at 4^oC.

Preparation of the honey, activator and eosin-Y solutions. The Ringer solution was mixed with 10% of Dimetyl Sulfoxide (DMSO) in the cryotube and it was kept at 4^oC. The commercial honey was then added into a mixture Ringer solution with 10% of DMSO, and was kept at 25^oC (Table 1). The activator solution was prepared by diluting 45 mM NaCl, 5 mM KCl, and 30 mM Tris with distilled water 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 distilled water up to 100 mL (WHO 1988).

Table 1

Composition of Ringer solution and commercial honey

Composition	*Honey solution concentration (%)*					
	0	*0.1*	*0.3*	*0.5*	*0.7*	*0.9*
Fish Ringer solution (mL)	100	100	100	100	100	100
Commercial honey (mL)	0	0.1	0.3	0.5	0.7	0.9

Preparation of 0.15M of phosphate buffer solution pH 6.8. The phosphate buffer solution was prepared by dissolving 5.34 g $Na_2HPO_4.2H_2O$ with distilled water up to 200 mL, and by dissolving 4.08 g KH_2PO_4 with distilled water up to 200 mL. $Na_2HPO_4.2H_2O$ solution was then added to KH_2PO_4 solution until the pH reached 6.8. The phosphate buffer solution was then kept at 4^oC.

Preparation of the Giemsa solution. The Giemsa solution was prepared by diluting one part of the Giemsa stock solution and 10 parts of the phosphate buffer solution pH 6.8. The mixed solution was then filtered by Whatman filter paper number one (WHO 1988).

Sperm collection. Six male donors weighing 2.5–4 kg were injected intra-muscularly with 0.2 mL of Ovaprim (Syndel Laboratories Ltd. Nanaimo, Canada) kg^{-1} body weight. After nine hours, the sperm was collected from individual male donor by abdominal gently stripping method (Muchlisin et al 2010) and placed in 2 mL vials (cryogenic storage vial, Nalgene Nunc International).

Sperm dilution. Fresh sperm suspension was diluted in the mix solution (diluents) containing Ringer solution, 10% of DMSO, and commercial honey (Table 2). The composition of mix solution was adopted from Salisbury et al (1985). The dilution ratio among the fresh sperm and mix solution was 1:3 according to Akcay et al (2004).

Table 2

Composition of sperm, 10% DMSO, Ringer solution and honey solution of experimental group

Composition	*Honey solution concentration (%)*					
	0	*0.1*	*0.3*	*0.5*	*0.7*	*0.9*
Semen/sperm (µL)	60	60	60	60	60	60
10% DMSO (µL)	24	24	24	24	24	24
Fish Ringer solution (µL)	156	0	0	0	0	0
Honey solution (µL)	0	156	156	156	156	156

* 0% honey solution as the control.

Cryopreservation process. The vials were labeled and allowed to equilibrate for 10 min in refrigerator (4^{o}C) to allow time for the sperm to be exposed to the diluents before freezing (Muchlisin & Siti-Azizah 2009). The tubes were then placed in the freezer (-34 ^{o}C) for 48 h based on modification method by Huang et al (2004).

Evaluation of motility, viability, and abnormality. After 48 h, the samples were thawed in a water bath at 40^{o}C for 2 min (Horvath et al 2003). The tube seals were opened and the samples were diluted in activator solution at dilution ratio 1:49 (sperm:activator solution, v/v) based on Sunarma et al (2007). Then, one drop (10 µL) of thawed sperm was placed onto improved Neubauer equipment for assessing sperm motility, and another drop (10 µL) for assessing sperm viability, while the remaining thawed sperm was used for assessing sperm abnormality.

Percentage of motility, viability, and abnormality were observed with a light microscope (Boeco BM-180 SP, Germany) connected with the digital eyepiece camera (MDCE 5ª) using Soft Imaging software analysis (Scopephoto 2.0.4). Spermatozoa motility was analyzed by subjective method (Rurangwa et al 2004), whereas spermatozoa viability and spermatozoa abnormality were analyzed by Salisbury et al (1985). The abnormalities were evaluated based on head morphology (swelled or rupture) and flagellum performance (present, absent or rupture). Fresh sperm color was observed visually, whereas sperm volume was measured by the vial with scale. Fresh sperm pH was measured by pH paper (pH range 5-10).

Statistical analysis. All data were subjected to analysis of variance (ANOVA), followed by Tukey's multiple comparison test. And the percentage data were Arcsine transformed prior to analysis (Sofyan & Werwatz 2001). The statistical analyses were performed using SPSS software version 15.0.

Results and Discussion. Fresh semen (FS) was milky white, pH 8.03±0.05, and 0.52±0.14 mL of volume per ejaculate (Table 3). Gourami spermatozoa had rounded head and a flagellum (tail). The viable or motile sperm showed green color (Figure 1) on

the sperm head, while the non-viable sperm showed pink or red color on the sperm head (Figure 1). Most of the spermatozoa in fresh milt have a normal structure. On the other hand, there were also many variations of abnormal head spermatozoa shown such as microcephalus, and spermatozoa without heads (Figure 2). Besides, there were many variations of abnormal flagellum (tail) spermatozoa shown in Figure 2. However, these abnormalities occur naturally. The percentages of sperm motility, viability and abnormality of fresh semen (FS) were 77.35±5.98%, 83.56±3.13% and 18.67±3.20%, respectively (Table 3), while, the percentages of sperm motility, viability and abnormality 48 hours after sub zero freezing can be seen in Table 4.

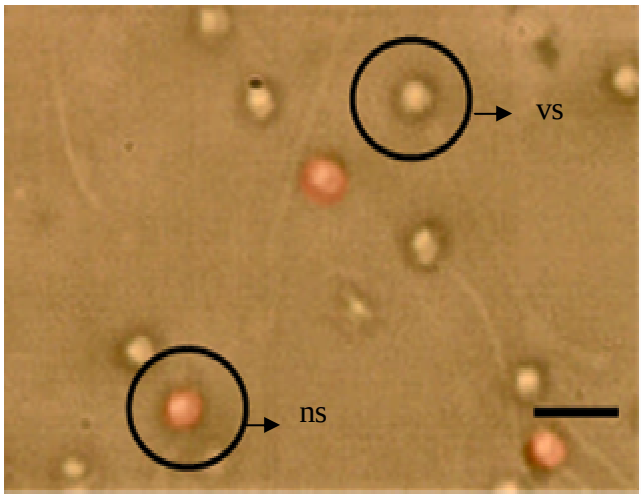

Figure 1. Spermatozoa viable (vs); spermatozoa non-viable (ns) with 10x40 magnification (96 x 96 dpi); bar = 10 µm.

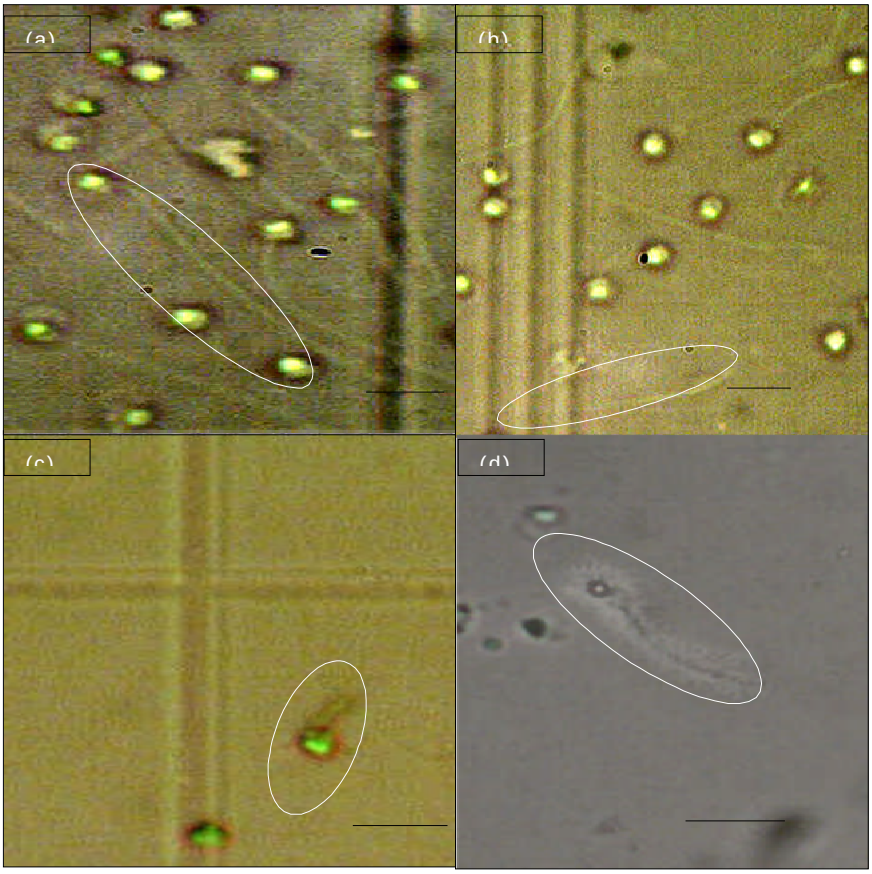

Figure 2. Normal spermatozoa (a); spermatozoa without head (b); cut tail spermatozoa (c); microcephalus spermatozoa (d); 10x40 magnification (96 x 96 dpi); bar = 10 µm.

Table 3

Fresh semen/sperm evaluation and spermatozoa analyses of gourami spermatozoa, before freezing

Color	*Volume (mL)*	*pH*	*Motility (%)*	*Viability (%)*	*Abnormality (%)*
Milky white	0.52±0.14	8.03±0.05	77.35±5.98	83.56±3.13	18.67±3.20

Values are means ± SD of four replicates.

Table 4

Motility, viability, and abnormality of gourami spermatozoa, 48 hours after sub-zero freezing

Paramater	*Honey solution concentration (%)*					
(%)	*0%*	*0.1*	*0.3*	*0.5*	*0.7*	*0.9*
Motility	59.68±12.05[a]	68.42±7.03[a]	74.43±12.03[a]	74.71±11.10[a]	80.48±7.18[b]	76.32±5.55[a]
Viability	71.33±6.59	76.50±6.06	75.17±6.18	82.67±4.18	74.83±7.11	77±6.72
Abnormality	17±4.34	17.67±3.83	16.67±5.57	29.75±0.5	28.25±1.5	26.25±1.89

Values are means + SD of four replicates. Mean values in the same raw with the same superscript are not significantly different ($p > 0.05$).

The ANOVA test showed that honey had a significant effect on the motility rate of gourami spermatozoa ($p < 0.05$). The thawed sperm cryopreserved in 0.7% of honey solution + 10% of DMSO showed the highest motility rate (80.48%) which was significantly different from the other groups ($p < 0.05$). But, there were no statistical differences in viability rate or abnormality rate ($p > 0.05$).

Among the tested natural cryoprotectants, the motility rate was the highest for the gourami spermatozoa cryopreserved in 0.7% of honey solution combined with 10% of DMSO (80.48%) compared to other fish species such as *Mystus nemurus* (58%; Muchlisin et al 2004), *Cyprinus carpio* (55%; Akcay et al 2004), and *Osteochilus hasselti* (63.33%; Sunarma et al 2007). On the other hand, the motility rate in the present study (80.48%) was lower than previous reported for *O. goramy* (80.98%; Abinawanto et al 2012b) and Java barb (83.23%; Abinawanto et al 2016).

The viability rate was the highest for the gourami spermatozoa cryopreserved in 0.5% of honey solution combined with 10% of DMSO (82.67%). However, there was no statistical difference among groups ($p > 0.05$). This result was higher compared to other species like Java barb (77.25%; Abinawanto et al 2009), *C. carpio* (20%; Withler 1982; 58%; Horton & Otto 1976), and to the same species (*O. goramy*) in other study (63.5%; Abinawanto et al 2011). Our previous study showed that the viability rate for the Java barb spermatozoa cryopreserved in 20% of skim milk combined with 5% of methanol was higher (83.23%; Abinawanto et al 2016) than the viability rate in the present study (82.67%).

There was no statistical difference between control (0%), 0.1%, 0.3%, 0.5%, 0.7%, and 0.9% of honey solution combined with 10% of DMSO in the abnormality rate ($p > 0.05$). However, the abnormality rate was the lowest for gourami spermatozoa cryopreserved in 0.3% of honey solution combined with 10% of DMSO (16.67%). The spermatozoa abnormality rate in the present study (16.67%) was the lowest compared to Java barb (26.25%; Abinawanto et al 2016) and gourami (29%; Abinawanto et al 2011).

Thawing procedures at 40^{o}C for 1-2 min were effective for 2 mL of cryogenic tubes. We choose 40^{o}C because this is easy to achieve using heating devices in our temperature conditions. DMSO as an intra-cellular cryoprotectant significantly improved motility of cryopreserved sperm. DMSO was employed as successful intra cellular cryoprotectant in several fish species, for example, *M. nemurus* (Muchlisin et al 2004; Muchlisin & Siti-Azizah 2009), *O. goramy* (Abinawanto 2011), and African catfish, *C. gariepinus* (Muchlisin et al 2015). Besides, methanol was also successful as an intra-cellular cryoprotectant in *Brachydanio rerio* (Muchlisin et al 2004); *Oreochromis mossambicus* (Harvey 1983); tilapia fish (Chao et al 1987); *C. carpio* (Horváth et al 2003); *O. goramy* (Abinawanto et al 2012a; Abinawanto et al 2012b), and *B. gonionotus* (Abinawanto et al 2009; Abinawanto et al 2013).

The present study showed that the combination of 0.7% of honey solution and 10% of DMSO is considered as an effective cryoprotectant for cryopreservation of gourami spermatozoa. Based on cryoprotectant activities, honey is considered as non-permeating or extra cellular cryoprotectant, while DMSO is permeating or intra cellular cryoprotectant (Muchlisin et al 2015). Therefore, DMSO can penetrate sperm cell membrane and enter the cytosol through its interaction with phospholipids of sperm cell membrane (Ogier de Baulny et al 1996). According to Thapliyal et al (2011), the permeating cryoprotectant can reduce the rate of water diffusion from cell to extra-cellular ice crystals. Therefore, it functions in sperm protection from cold and heat shock efficiently, during freezing and thawing. Regarding a natural cryoprotectant agent, the present study revealed that 0.7% honey solution was the optimal concentration for preserving motility rate of gourami spermatozoa to other natural cryoprotectants investigated. It has been well documented that honey solution becomes an effective cryoprotectant for cryopreservation of sperm in several fish species, for example, for *C. gariepinus* (Muchlisin et al 2015) and *O. hasseltii* (Sunarma et al 2007). Honey contains sucrose, glucose, fructose, protein, and minerals (Hacettepe 2008) which can act as protective agent extracellularly (Abinawanto et al 2009; Abinawanto et al 2012a).

Conclusions. The combination of 0.7% of honey solution and 10% of DMSO was the most optimal concentration which showed the highest motility rate of gourami, *Osphronemus goramy* spermatozoa. However, the fertilization study is yet to be clarified for further investigations.

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