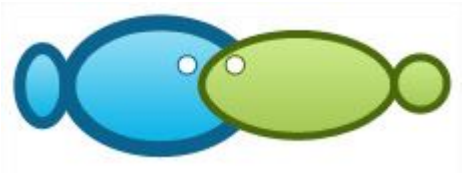

Sperm viability and quantity of mud crab, *Scylla tranquebarica* in different cryoprotectants

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Abstract. The aim of the present study was to determine the optimum concentrations of different cryoprotectants and to determine the sperm quantity with different durations of exposure for *S. tranquebarica*'s sperm. The body weight of *S. tranquebarica* was 280-350 g. In the present study, male *S. tranquebarica* were dissected out and got the sperm to determine its viability and quantity using six types of cryprotectants (glycerol, glycine, methanol, dimenthyl sulfoxide (DMSO), ethylene glycol (EG) and proline). The sperm was exposed with different durations of 5, 15, 30 and 60 min at room temperature (25°C). For the cryoprotectant glycine 10%, there was the highest mean sperm viability with 84.75±1.01% (exposure at 60 min). Meanwhile, 15% proline was the lowest mean sperm viability with 15.39±0.39% after exposure for 60 min at room temperature. There were significant differences between the duration of exposure for some types of cryoprotectants (p-value < 0.05). As a conclusion, 10% glycine was the best cryoprotectant of mud crab, *S. tranquebarica*. As a recommendation, *S. tranquebarica*'s sperm should be preserved in the cold conditions such as -20°C, -80°C and -196°C (liquid nitrogen) to determine the sperm viability and quantity for further breeding programs and biochemical changes.

Key Words: *Scylla tranquebarica,* cryoprotectant, sperm, concentration.

Introduction. The aquaculture industry is gaining importance and contributes significantly towards Malaysia's economy in recent years. The mud crab *Scylla tranquebarica* has been economically important for the Malaysian market as this species can be found almost at every offshore and coastline of Malaysia (Azra & Ikhwanuddin 2016). The high value of meat and tastes gives the species a placed among other types of crustaceans in the market (Ikhwanuddin et al 2014a). Besides, mud crab are considerably in demand as a quality food owing to their size, meat content and delicate flavour (Azra & Ikwanuddin 2016) and therefore, command high prices in domestic and international markets (Fatihah et al 2017). The production of mud crab in Malaysia has started way back in the 1990s. This included the farming of mud crab in cage culture and pen culture (Tan 1999).

Currently, mud crab farming has gained much attention in Malaysia. Nevertheless, most of these farming activities involve only crab fattening and the production of soft-shelled crabs from the wild (Ikhwanuddin et al 2013; Waiho et al 2015). Furthermore, increasing market demand led to the constraint supply of seed stocks caused by over exploitation (Ikhwanuddin et al 2015a). Besides, large scale aquaculture of mud crabs is presently limited owing to very low success rate in the hatchery production of juveniles (Quinitio & Parado-Estepa 2008; Holme et al 2009; Waiho et al 2015; Waiho et al 2018). One of the major constraints with supplies from wild caught seed is sperm quality. Most of the seed caught in the wild do not provide sufficient sizes and healthy sperm for hatchery production thus, reducing the mud crab yield production (Kosuge 2001; Ikhwanuddin et al 2014b). Another problem associated with wild caught seed is diseases.

Wild caught seed is most susceptible to disease and can transmit it to other culture in the hatchery.

Nowadays, cryopreservation has been seen as an alternative source of increasing the production of mud crab. By preserving the sperm, hatchery can continuously supply the good quality of sperm whenever there is a good quality of eggs from spawning female mud crab (Ikhwanuddin et al 2014a). Study by Ikhwanuddin et al (2014b) showed that the sperm viability assessment on orange mud crab, *Scylla olivacea* without cryoprotectants that maintained at 2^{o}C. Later, the previous study by Ikhwanuddin et al (2015b) showed that DMSO gave the greatest protection for sperm cells of *S. olivacea* while the effectiveness of DMSO was influenced by the sperm density. Besides, there are a few studies on cryoprotectants for the sperm in the other crustacean's species such as edible rock lobster, *Panulirus homarus* (Sasikala & Meena 2009), banana shrimp, *Penaeus merguiensis* (Memon et al 2012), white shrimp, *Litopenaeus vannamei* (Castelo-Branco et al 2015) and mud spiny lobster, *Panulirus polyphagus* (Fatihah et al 2016). Thus, the objectives in the present study are to determine the optimum concentrations of different cryoprotectants and in the same time determining the sperm quantity after exposure for *S. tranquebarica*'s sperm.

Material and Method

Study site and sample collection. The research study was done from January to March 2017 in the Shrimp Hatchery, Universiti Malaysia Sabah (UMS). A total of 40 matured male broodstock of *S. tranquebarica* were bought from the market in Tuaran and Kota Kinabalu, Sabah, Malaysia. Prior to dissection, the body weight (BW) and carapace width (CW) of the crabs were measured. The BW of *S. tranquebarica* which was used in the study had the range 280-350 g. The sperm was obtained using dissection method. The carapace of *S. tranquebarica* was opened and the internal organ was dissected separating the testes (Figure 1). The testes were homogenized manually using a mortar and pestle before the sperm was separated and collected. Immediately after that, the sperm was kept within an insulating box filled with ice to maintain the sperm quality.

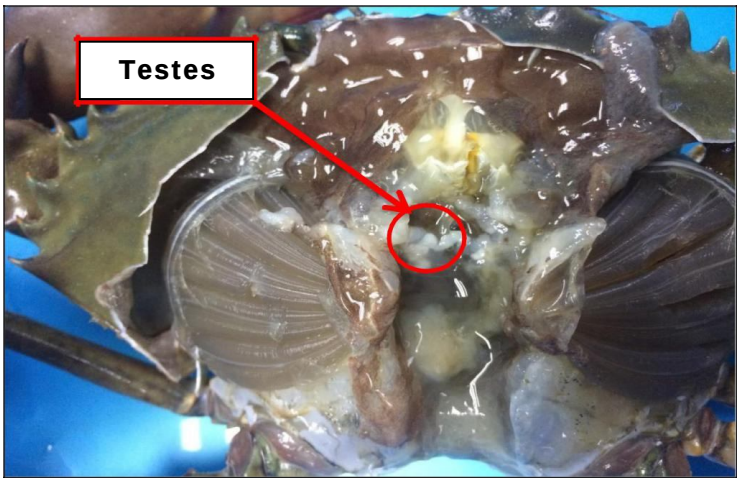

Figure 1. The carapace of *Scylla tranquebarica* was opened and the internal organ was dissected separating the testes.

Preparation and dilution of extender and cryoprotectants. There was the list of materials to prepare the Calcium Free Saline (Ca-F saline): 21.63 g NaCl, 1.12 g KCl, 0.53 g H_3BO_3, 0.19 g NaOH and 0.493 g $MgSO_4$ in a 1L sterile distilled water (adjusted to pH 7.4 with NHCl) (Vuthiphandchai et al 2007; Fatihah et al 2016). After sperm collection, the sample was diluted with Ca-F saline with the ratio of 1:3 (sperm: extender). A total of 3000 µL Ca-F saline was used to dilute the *S. tranquebarica* sperm. The sample was kept in microcentrifuge tube under room temperature (25°C). Six types of cryoprotectants were prepared, which are glycerol, glycine, methanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and proline. Each cryoprotectant was prepared

with different concentrations which are 5, 10, 15 and 20 %. The sperm was diluted or exposed with each concentration (5, 10, 15 and 20 %) of cryoprotectants at ratio of 1:3, which means that 200 µL of sperm was exposed with 600 µL of cryoprotectant. Each concentration was used in triplicates for evaluation of the sperm viability.

Experimental design. The preparation of extender and cryoprotectants was done a day before the collection of samples. The ratio for the cryprotectants and the sperm was 1:3 (suspended sperm: cryoprotectants). Then, the sperm was diluted with cryprotectants and was kept inside microcentrifuge tube. After that, the sperm viability was observed under compound microscope (Leica DM750) in the intervals of 5, 15, 30 and 60 min.

Sperm count. To evaluate the sperm viability after the dilution with cryoprotectants, the eosin-nigrosin staining method was used (Zaneveld & Polakoshi 1977; Jeyalactumie & Subramoniam 1989; Memon et al 2012; Fatihah et al 2016). A smear was prepared by mixing 300 µL sperm with one drop of 5% eosin and two drops of 10% nigrosin inside microcentrifuge tube and shaken to mix properly. Then, a drop of 10 µL mixture was smeared on haemocytometer slide and examined under compound microscope (40 x magnifications).

To calculate the percentage of live sperm, the number of live and dead sperms was counted. The viability and quantity of the sperm were observed through the light microscope (Vuthiphandchai et al 2007). The live sperm was observed as unstained, whereas the dead sperm was observed as pink or red stained against the eosin-nigrosin background (Akarasanon et al 2004; Bart et al 2006; Fatihah et al 2016). Sperm viability was determined based on the equation:

$$\text{Sperm viability} = \frac{\text{Observed number of live sperm}}{\text{Total number of sperm observed (live + dead)}} \times 100\%$$

Besides, for the sperm quantity calculation, the totals of sperm present in five of the 25 squares on the haemocytometer with complete test scope were tallied. The mean sperm quantity in the 25-square grid (0.1 µL) was multiplied by 10^4 cells mL^{-1} based on the following equation by Hala et al (2009), Fatihah et al (2014a, 2014b):

$$\text{Sperm quantity (sperm } mL^{-1}) = \text{mean count of 5 total squares} \times 25 \text{ (25 squares)} \times (10^4 \text{ cells/mL}) \times \text{dilution factor of sperm}$$

Data analysis. The effects of different concentrations for cryoprotectants in mud crab, *S. tranquebarica* were analysed using SPSS (Statistical Package for Social Science) and PRIMER v6. One-way analysis of variance (ANOVA) was chosen to measure the significance of different concentrations for cryprotectants over duration of exposure. Cluster analyses were conducted to determine the similarity in the effects of different cryoprotectants in different concentrations.

Results. From the results, the highest mean sperm viability was 84.75±1.01% in 10% glycine and the lowest mean sperm viability was 15.39±0.39% in 15% proline (Table 1). The highest mean sperm quantity from 5 min until 60 min were 5% glycerol (4.18×10^6 cells mL^{-1} (5 min) and 2.53×10^6 cells mL^{-1} (60 min)), and 10% glycine (4.00×10^6 cells mL^{-1} (5 min) and 3.15×10^6 cells mL^{-1} (60 min)) (Table 2). The similarity of the samples with different cryoprotectants and concentrations for 60 min exposure at room temperature was showed in Figure 2 and the distance of the samples with different cryoprotectants and concentrations with different duration (5, 15, 30 and 60 min) was showed in Figure 3.

Table 1

Mean *S. tranquebarica* sperm viability (%) after exposure to 0, 5, 10, 15 and 20% in DMSO, EG, proline, glycerol, glycine and methanol respectively in different durations (5, 10, 15 and 60 min) (mean±standard deviation)

Cryoprotectants	*Time (min)*			
	5 min	*15 min*	*30 min*	*60 min*
5% DMSO	66.94±0.85	66.88±1.01	59.22±0.34	58.65±0.39
10% DMSO	84.26±1.48	73.18±0.34	72.87±0.84	65.78±0.93
15% DMSO	83.75±0.97	74.59±1.13	63.99±0.22	63.26±1.49
20% DMSO	66.08±0.45	63.25±0.66	48.64±1.77	44.41±0.66
5% EG	62.87±0.32	49.75±0.82	38.97±0.73	34.49±0.51
10% EG	60.27±0.28	44.48±0.53	38.37±0.34	28.57±0.74
15% EG	64.33±0.72	56.46±0.49	45.83±0.21	24.11±0.15
20% EG	70.56±0.09	46.55±0.22	36.79±0.19	22.18±0.30
5% Proline	73.94±0.09	58.66±0.84	38.39±0.62	36.23±0.17
10% Proline	67.76±0.25	57.96±0.16	53.19±0.54	36.32±0.29
15% Proline	61.05±0.09	49.23±0.49	34.62±0.35	15.39±0.39
20% Proline	48.06±0.15	36.12±0.51	29.17±0.32	21.73±0.35
5% Glycerol	93.51±0.53	90.37±0.52	85.59±1.80	78.3±2.15
10% Glycerol	91.06±0.86	89.67±0.35	79.64±0.85	70.1±0.58
15% Glycerol	96.32±0.52	87.85±1.00	83.6±2.17	73.13±2.06
20% Glycerol	89.89±0.93	85.32±0.47	83.91±0.65	62.66±0.94
5% Glycine	91.02±0.58	83.36±1.21	80.43±0.85	70.48±2.03
10% Glycine	89.72±1.76	85.71±1.93	85.17±2.29	**84.75±1.01[a]**
15% Glycine	94.3±0.61	80.62±1.13	72.44±2.73	71.99±1.35
20% Glycine	86.84±1.06	65.52±1.68	59.55±1.17	55.41±1.20
5% Methanol	79.95±0.82	75.37±1.66	73.15±1.33	71.09±1.80
10% Methanol	88.47±1.01	88.12±0.97	80.35±0.28	69.07±1.02
15% Methanol	95.92±0.08	90.2±0.49	82.13±1.89	72.31±2.05
20% Methanol	97.67±0.42	85.29±0.79	71.35±1.16	59.43±0.57

*Superscript a (with bold) = the highest mean sperm viability (%) after exposure for 60 min.

Table 2

Mean *S. tranquebarica* sperm quantity (cells mL^{-1}) after exposure to 0, 5, 10, 15 and 20% in DMSO, EG, proline, glycerol, glycine and methanol respectively in different durations (5, 10, 15 and 60 min)

Cryoprotectants	*Time (min)*			
	5 min	*15 min*	*30 min*	*60 min*
5% DMSO	$3.93x10^6$	$3.74x10^6$	$1.91x10^6$	$1.10x10^6$
10% DMSO	$2.64x10^6$	$2.04x10^6$	$0.91x10^6$	$0.78x10^6$
15% DMSO	$1.76x10^6$	$0.81x10^6$	$0.45x10^6$	$0.56x10^6$
20% DMSO	$0.65x10^6$	$0.56x10^6$	$0.45x10^6$	$0.38x10^6$
5% EG	$1.56x10^6$	$1.24x10^6$	$0.65x10^6$	$0.53x10^6$
10% EG	$1.61x10^6$	$0.71x10^6$	$0.58x10^6$	$0.36x10^6$
15% EG	$1.11x10^6$	$0.70x10^6$	$0.68x10^6$	$0.29x10^6$
20% EG	$1.11x10^6$	$0.68x10^6$	$0.56x10^6$	$0.18x10^6$
5% Proline	$1.01x10^6$	$0.81x10^6$	$0.41x10^6$	$0.36x10^6$
10% Proline	$1.03x10^6$	$0.83x10^6$	$0.78x10^6$	$0.30x10^6$
15% Proline	$1.03x10^6$	$0.69x10^6$	$0.43x10^6$	$0.21x10^6$
20% Proline	$0.96x10^6$	$0.54x10^6$	$0.49x10^6$	$0.21x10^6$
5% Glycerol	$4.18x10^6$	$3.60x10^6$	$3.09x10^6$	$2.53x10^6$
10% Glycerol	$3.13x10^6$	$2.96x10^6$	$2.77x10^6$	$2.43x10^6$
15% Glycerol	$3.33x10^6$	$3.27x10^6$	$2.98x10^6$	$2.86x10^6$
20% Glycerol	$3.46x10^6$	$3.25x10^6$	$3.01x10^6$	$1.46x10^6$
5% Glycine	$3.47x10^6$	$3.17x10^6$	$2.96x10^6$	$2.44x10^6$
10% Glycine	$4.00x10^6$	$3.58x10^6$	$3.36x10^6$	**$3.15x10^{6\,(a)}$**
15% Glycine	$3.39x10^6$	$3.25x10^6$	$2.96x10^6$	$2.66x10^6$
20% Glycine	$3.27x10^6$	$2.59x10^6$	$2.47x10^6$	$2.40x10^6$
5% Methanol	$3.45x10^6$	$3.11x10^6$	$2.44x10^6$	$2.13x10^6$
10% Methanol	$3.75x10^6$	$3.27x10^6$	$3.21x10^6$	$2.83x10^6$
15% Methanol	$3.45x10^6$	$3.41x10^6$	$3.20x10^6$	$2.93x10^6$
20% Methanol	$3.57x10^6$	$3.38x10^6$	$3.05x10^6$	$2.21x10^6$

*Superscript a (with bold) = the highest mean sperm quantity (cells mL^{-1}) after exposure for 60 min.

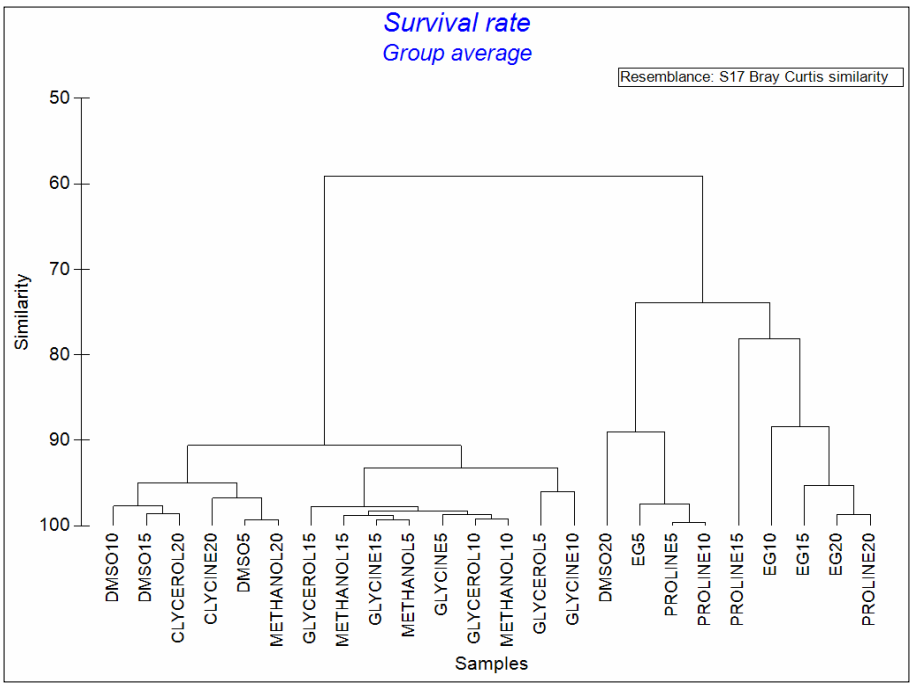

Figure 2. The similarity of the samples with different cryoprotectants and concentrations for 60 min exposure at room temperature.

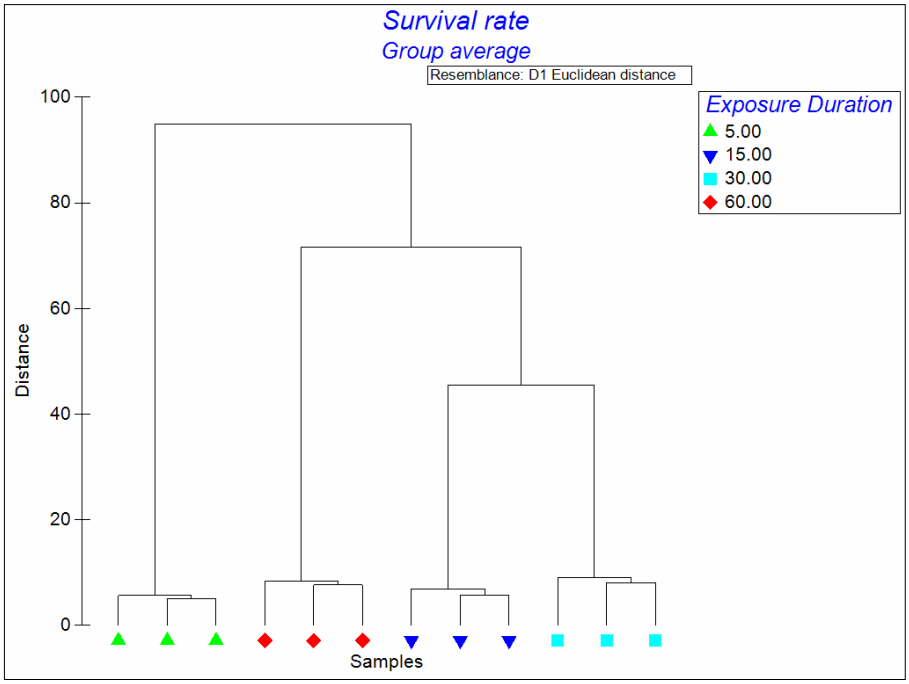

Figure 3. Cluster analysis on the survival rate of the sperm in subjected to different exposure duration (5, 15, 30 and 60 min).

At the 65% of similarity, the results of the cluster analysis demonstrated that the survival rates of the *S. tranquebarica*'s sperms in different cryoprotectants were divided into two groups. The first group showed an extreme low survival rate that ranged from 15.39 to 44.41%. The lowest mean sperm viability after exposure for 60 min were with ranged 15.39 to 28.57% in 15% proline, 20% proline, 10% EG, 15% EG and 20% EG. For the cryoprotectants of 5% EG, 5% proline and 20% DMSO, the mean sperm viability was 34.49 to 44.41%. The second group that clustered together (Figure 2) indicated higher survival rates (subjected to different types and concentrations of cryoprotectants). Cryoprotectants such as 5% DMSO, 20% glycine and 20% methanol recorded with the

mean sperm viability ranged from 55.41 to 59.43%. Besides that, higher mean sperm viability was found in 10% DMSO, 15% DMSO and 20% glycerol (62.66 to 65.78%). Furthermore, for the cryoprotectants of 5% methanol, 10% methanol, 15% methanol, 5% glycine, 15% glycine and 10% glycerol, the mean sperm viability was 70.48 to 71.99%. The highest mean sperm viability were in 15% glycerol (73.13%), 5% glycerol and 10% glycine with ranged 78.30 to 84.75%.

For the present results, there were significant differences between the duration of exposure for some types of cryoprotectants (p-value < 0.05). However, certain cryoprotectants did not show any significant difference between certain exposure durations. In the 5% DSMO, no significant difference was recorded between 5 and 15 minutes (p-value = 0.999); 30 and 60 minutes (p-value: 0.758). In a different concentration of 10% DMSO, the results showed no significant difference between 15 and 30 minutes of exposure (p = 0.979) as compared to 15% DMSO (30 and 60 minutes: p = 0.835). Besides, the cryoprotectants of 5% glycerol and 10 % glycerol showed no significant difference between 5 and 15 minutes with p = 0.109 (5% glycerol) and p = 0.145 (10% glycerol) while the results showed no significant difference for 20% glycerol between 15 and 30 minutes with p = 0.194. For the cryoprotectants of 10% glycine, the results also showed no significant difference between 15, 30 and 60 min (p = 0.911 to 0.991) and the results for 15% glycine showed no significant difference between 30 and 60 min (p = 0.986). In the different concentration of 5% methanol, the results showed no significant difference between 15, 30 min (p = 0.312) and 60 min (p = 0.363) while for 10% methanol, there was also no significant difference between 5 and 15 min (p = 0.960).

Discussion. In the present study, *S. tranquebarica*'*s* sperm was able to survive during exposure for 60 min at room temperature (25°C). In any case, the concentration of cryoprotectants and the exposure time intervals influenced the sperm viability at room temperature (25°C). Extender is a salt buffer solution that used during the sperm collection for cryopreservation and intermediate to dilute sperm and gain more volume of diluted sperm for artificial breeding. In addition, a cryoprotectant is a solution which consolidated into an extended sperm to maintain a strategic distance from any harm for the sperm during cooling, freezing and thawing of the sperm (Alawi et al 1995). The present study used Ca-F saline as an extender and the previous studies were also used Ca-F saline as the extender to protect the sperm of the giant tiger prawn, *P. monodon* (Vuthiphandchai et al 2007), banana shrimp, *P. merguiensis* (Memon et al 2012) and mud spiny lobster, *P. polyphagus* (Fatihah et al 2015, 2016).

Previous studies showed that glycerol and DMSO are commonly used for cryopreservation of the sperm crustacean (Anchordoguy et al 1988; Diwan & Joseph 2000; Lezcano et al 2004; Kang et al 2009; Sasikala & Meena 2009). Furthermore, DMSO has been claimed as the best cryoprotectant for the crustacean species (Ikhwanuddin et al 2014a). In the present study, six cryoprotectants (glycerol, glycine, methanol, DMSO, EG and proline) were used in sperm viability and quantity after exposure at room temperature (25^{o}C). Cryoprotectants provide protections for the sperm cells but increased concentrations of cryoprotections may exert toxicity to the sperm cells. Therefore, the optimum concentration depends on the level of toxicity and capacity of cryoprotectants to protect the sperm cells. The balance between solute influx and water outflow during freezing was determined by cryoprotectants exposures to the sperm cells. Hence, the cryoprotectants with low level of toxicity and high permeability were required. In the present study, the optimum level concentrations of cryprotectants and durations of exposure were determined in *S. tranquebarica's* sperm.

Furthermore, sperm quality influenced by the quality of the wild broodstock. Sperm cells appeared uniformly spherical in shape were discharged from the spermatophores located in the vas deferens (Ikhwanuddin et al 2014b). Different equilibration times (10, 20, 30 and 60 min) were tested with several cryoprotectants for the *Penaeus monodon* sperm (Vuthiphandchai et al 2007). The survival of sperm cells in *S. tranquebarica* showed the similar reductions of the sperm were exposed with different concentration of cryoprotectants over 5, 15, 30 and 60 min. In the present study, 10%

glycine showed the higher sperm viability compared to other concentrations. From a previous study, the optimum level of concentrations in glycerol and methanol was 5% in cryopreservation of *S. olivacea* sperm and showed the higher sperm viability in 5% methanol (Ikhwanuddin et al 2014b). Thus, glycerol and methanol showed the different effect on sperm viability with different species.

At 60 min, all treatments exposed with cryoprotectants showed a significant decreased in the number of sperm observed. The decreased of sperm were due to the level of toxicity in cryoprotectants exert of sperm cells. *S. tranquebarica's* sperm exposed with 10% glycine showed the higher sperm viability with 84.75±1.01% compared to 5% glycine, 15% glycine and 20% glycine. Meanwhile, the sperm cells exposed with 15% methanol showed higher sperm viability with 72.31±2.05% compared to 5% methanol, 10% methanol and 20% methanol. Other previous study showed that 5% glycerol, 5% methanol and 5% EG were 71.5±12.7%, 70.1±14.6%, and 63.3±11.2% respectively in sperm viability of banana shrimp, *P. merguiensis* exposure at room temperature (25^{o}C) (Memon et al 2012). The present study showed that 10% glycine was more effective in sperm cells protection of *S. tranquebarica*. These results indicated that different cryoprotectants with different concentrations showed significant effects on sperm viability and each cryoprotectant provided different results in different species of crustaceans.

The use of eosin-nigrosin staining method is to observe the sperm motility and sperm viability. Eosin-nigrosin staining technique was used for staining the live and dead sperm of *P. merguiensis* (Memon et al 2012). Furthermore, Ikhwanuddin at al (2014b) also used the eosin-nigrosin staining method in cryopreservation of orange mud crab, *S. olivacea*. Consequently, these methods have been utilized to observe sperm quantity and sperm viability (Memon et al 2012; Fatihah et al 2016).

Conclusions. The effect of sperm viability was influenced by different types of cryoprotectants and concentrations. Different cryoprotectants showed different times of penetration to the sperm cells meanwhile different concentrations showed different level of toxicity and cells protection in species. Besides, sperm quantity was decreased when the exposure of sperm was increased and the highest sperm quantity in the present study was 4.18 x 10^6 cells mL^{-1}. Based on the present study, the best cryoprotectant for *S. tranquebarica* was 10% glycine with 84.75±1.01% which showed highest percentage of live sperm over the durations of 60 min. Future studies should include the freezing of sperm in liquid nitrogen and the effect of different concentrations of cryprotectants in post-thaw. However, a complete fertilization and hatching percentage should be the future studies about this cryopreservation to determine most effective cryoprotectant.

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