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Development of a sperm cryopreservation protocol for the mud spiny lobster, *Panulirus polyphagus*

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ABSTRACT

This study aimed to develop a cryopreservation protocol for sperm of the mud spiny lobster, *Panulirus polyphagus*. Sperm of *P. polyphagus* were successfully cryopreserved using a protocol with cooling periods of 15 min per temperature, 25, 20, 16, 4, 2, −4, −20, −80, and −150 °C, followed by immediate storage in liquid nitrogen (at −196 °C). The efficacy of the cryopreserved protocol was determined by assessing the viability of sperm. The optimal thawing temperature for cryopreservation of sperm was 26 °C for 30 s, with a viability rate of 76.09% ± 7.81. At room temperature, −20 and −80 °C, 10% glycine provided the highest percentage of sperm viability at 91.87 ± 2.03% (5 min at room temperature), 91.31 ± 2.65% (6 h at −20 °C) and 75.88 ± 10.81% (6 h at −80 °C). In conclusion, we developed a protocol (Protocol I) for the successful cryopreservation of *P. polyphagus* sperm using Ca-F saline as an extender and 10% glycine as a cryoprotectant.

Statement of relevance: This article is suitable with Aquaculture Journal because the findings will give a big contribution in mud spiny lobster aquaculture. Mud spiny lobster seedling especially *P. polyphagus* is getting less landing every year, new approach needed in ensuring enough supplies of *P. polyphagus* to be available. The content in this article would help developing the research innovation for the hatchery production of *P. polyphagus* to increase.

1. Introduction

The mud spiny lobster, *Panulirus polyphagus*, is in high demand on the international market. In Hong Kong, Taiwan and Singapore, the spiny lobster *Panulirus* sp. occupies a niche of live reef fish trade markets (Biusing & Lin, 2004). The commercial-scale hatchery production of spiny lobster pueruli and juveniles is difficult (Phillips and Kittaka, 2000), and studies of the restocking of spiny lobsters remain limited (Herrnkind et al., 1997; Phillips and Evans, 1997). The predominant problems facing *P. polyphagus* breeding include low survival rates of the pueruli larval stage, the delicate maintenance of pueruli and juveniles, and difficulty in obtaining mature broodstock, particularly *P. polyphagus* males (Fatihah et al., 2014a, 2014b; Ikhwanuddin et al., 2014a). Thus, artificial insemination, discriminating crossbreeding, domestication and conservation of stocks, and sperm cryopreservation techniques will improve the cultivation of *P. polyphagus*. Furthermore, sperm cells are preferable for cryopreservation over eggs due to their large quantities, ease of collection and overall feasibility (Sasikala and Meena, 2009).

Previous studies have discovered the feasibility of sperm cryopreservation in species such as the horseshoe crab, *Limulus polyhemus* (Behlmer and Brown, 1984), the fresh water prawn, *Macrobrachium rosenbergii* (Chow et al., 1985), the Ridgeback rock shrimp, *Sicyonia ingentis* (Anchordoguy et al., 1988), the mud crab, *Scylla serrata* (Bhavanishankar and Subramoniam, 1997; Chow et al., 1985), the Whiteleg shrimp, *Litopenaeus vannamei* (Dumont et al., 1992), the Chinese white shrimp, *Penaeus chinensis* (Ke and Cai, 1996), the Giant tiger prawn, *Penaeus monodon* (Bart et al., 2006; Vuthiphandchai et al., 2007), the abalone, *Haliotis diversicolor*, the oyster, *Crassostrea virginica* (Paniague-Chavez and Tiersch, 2001) and the *Penaeus merguiensis* (Memon et al., 2012). Indeed, most studies have focused on optimizing cooling rates for invertebrate gamete cryopreservation rather than on the process of cryopreservation (Anchordoguy et al., 1988; Jeyalectumic and Subramoniam, 1989; Bart et al., 2006; Vuthiphandchai et al., 2007).

Furthermore, sperm cryopreservation techniques are well developed in marine invertebrates such as the sea urchin, *Tetrapigus niger* (Barros et al., 1996; Adams et al., 2004), the Pacific oyster, *Crassostrea gigas* (Usuki et al., 1997; Sansone et al., 2002), the abalone, *H. diversicolor* (Matunaga et al., 1983; Gwo et al., 2002), the sea cucumber, *Apostichopus japonicus* (Shao et al., 2006) and the Japanese pearl oyster, *Pinctada fucata martensii* (Narita et al., 2008) because these

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preserved gametes exhibit high fertilization rates. In contrast, this study attempts to identify suitable extenders (Ca-F saline, Ringer's solution and phosphate buffer), cryoprotectants (Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), glycerol, methanol and glycine), cooling rates and thawing temperatures for the cryopreservation of *P. polyphagus* sperm.

2. Materials and methods

2.1. P. polyphagus lobster brood stock samples

Eighty brood stock mature *P. polyphagus* males with the carapace lengths ranging from 6.02 to 8.02 cm (Ikhwanuddin et al., 2014b) were collected from Tanjung Sedili Kecil (1° 51′ N and 104° 09′ E), Kota Tinggi, Johor and maintained for 12 months. There were 12 groups with four replicates in each group for cryopreservation protocol development experiments. Three treatments with four replicates were used to determine the optimal extender. Five treatments with four replicates were used to determine the optimal cryoprotectants. To determine the optimal thawing temperature, 10 treatments with four replicates each were assessed. All of the experiments in this study were performed from January 2013 to January 2014.

3. Experimental design

3.1. Determination of suitable extenders

The following three types of extenders were used in this study: Calcium-free saline (Ca-F saline), Ringer's solution and phosphate buffer solution. Ca-F saline was prepared by mixing 21.63 g of NaCl, 1.12 g of KCl, 0.53 g of H_3BO_3, 0.19 g of NaOH and 4.39 g of $MgSO_4 \cdot 7H_2O$ in 1 L of sterile distilled water (adjusted to pH 7.4 with 1 N HCl) (Vuthiphandchai et al., 2007). Ringer's solution was prepared by diluting 7.2 g of NaCl, 0.17 g of $CaCl_2$ and 0.37 g of KCl in 1 L of sterile distilled water followed by adjustment to pH 7.3–7.4 (Cold Spring Harbor Protocols, 2008). The phosphate buffer solution was prepared by mixing 2.86 g of NaH_2PO_4 and 0.2 g of KH_2PO_4 in 1 L of sterile distilled water. An additional 8 g of NaCl was added to the mixture to adjust its pH to 7.4 (Nugroho et al., 2004).

Sperm samples were obtained after the testes of mature male *P. polyphagus* were dissected and removed. The extender was added to the testes and homogenized manually using a mortar and pestle. Whitish matter of the sperm was expelled out from the lobster's testes. Immediately, the sperm were transferred into 2-mL cryovials, and extenders (Ca-F saline, Ringer's solution and phosphate buffer) were added at ratio of 1:3. Each cryovial was incubated for 5, 15, 30 to 60 min at 25 °C (room temperature). For each incubation, 10 μL of sperm were transferred onto a Neubauer hemocytometer for counting, and an additional 50 μL of sperm was stained with 5% eosin and 10% nigrosin for microscopic observation (Leica DM750) to assess the viability of the sperm (modified from Vuthiphandchai et al., 2007).

3.2. Determination of suitable cryoprotectants

In this study, the following five cryoprotectants were tested; Dimethyl sulfoxide (DMSO) (Merck), Ethylene glycol (EG) (Merck), glycerol (Merck), methanol (Sigma) and glycine (Merck). Ca-F saline was selected as the extender during this experiment because it was most appropriate for the spermatophore cryopreservation of the Banana shrimp, *P. merguiensis* (Memon et al., 2012) and the Orange mud crab, *Scylla olivacea* sperm (Ikhwanuddin et al., 2014c). In addition, 5, 10, 15 and 20% cryoprotectant solutions were prepared by diluting the previously mentioned cryoprotectants with Ca-F saline.

3.3. Determination of sperm viability and quantity

The sperms were immediately transferred into cryovials (2 mL) and the 5, 10, 15 or 20% cryoprotectant solutions were added at a ratio of 1:3. After incubation periods of 5, 15, 30 and 60 min at 25 °C and of 6, 12 and 24 h at −4, −20 and −80 °C, sperm from each experimental incubation were counted using a Neubauer hemocytometer whereas another group of sperms were stained with 5% eosin and 10% nigrosin. The viability of the sperm was subsequently evaluated through microscopic examination (Leica DM750) (modified from Vuthiphandchai et al., 2007). Live (viable) sperm were appeared as unstained against the blue nigrosin background, whereas dead sperm exhibited pink- or red-stained membranes (Akarasanon et al., 2004; Bart et al., 2006; Ikhwanuddin et al., 2014c) (Fig. 1).

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

For quantitation of the sperm, the amount of sperm present in five of the 25 squares on the hemocytometer with complete sample coverage were counted. The mean sperm counts in the 25-square grid (0.1 μL) was multiplied by 10^4 cells/mL (Hala et al., 2009; Fatihah et al., 2014a, 2014b).

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

3.4. Development of cryopreservation protocol (estimation of cooling rates)

The suspension (testes homogenized with 5 mL of Ca-F saline) and cryoprotectant and 10% glycine were mixed at a ratio of 1:3 in cryovials, which were capped, cooled and subjected to 12 different cooling protocols (A–L) (Table 1). The cryovials were capped and chilled for 15 min at 25, 20, 16, 4, 2 and −4 °C (1st stage - air conditioned room and refrigerator), −20 to −80 °C (2nd stage - deep freezer), −100 to −150 °C (3rd stage - liquid nitrogen vapor), followed by storage at -196 °C (4th stage - liquid nitrogen) for 24 h (Table 1 (Protocol I)) (Fatihah et al., 2015).

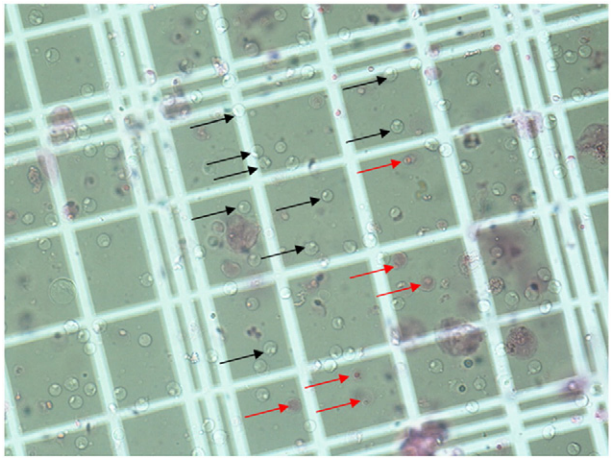

Fig. 1. Black arrows indicate live unstained sperm (viable sperm) cells, whereas the red arrows indicate dead sperm cells of *P. polyphagus*. The sperm cells were observed under 40× magnification.

Table 1
Different cooling rate estimations for the cryopreservation of *P. polyphagus* sperm.

Protocol	Time (min)	Temperature (°C)									
A	5	25	20	16	4	2	−4	−20	−80	−150	−196
B	5	25	20	16	4	2	−4	−20	−150	−196	–
C	5	25	20	16	4	2	−4	−80	−150	−196	–
D	5	25	20	16	4	2	−4	−150	−196	–	–
E	10	25	20	16	4	2	−4	−20	−80	−150	−196
F	10	25	20	16	4	2	−4	−20	−150	−196	–
G	10	25	20	16	4	2	−4	−80	−150	−196	–
H	10	25	20	16	4	2	−4	−150	−196	–	–
I	15	25	20	16	4	2	−4	−20	−80	−150	−196
J	15	25	20	16	4	2	−4	−20	−150	−196	–
K	15	25	20	16	4	2	−4	−80	−150	−196	–
L	15	25	20	16	4	2	−4	−150	−196	–	–

3.5. Determination of sperm thawing temperature

Sperm removed from the crushed testes were mixed with 10% glycine and chilled at 25, 20, 16, 4, 2 to −4 °C (1st stage – 15 min), −20 to −80 °C (2nd stage - 15 min), −100 to −150 °C (3rd stage – 15 min) before storage in liquid nitrogen at −196 °C (4th stage – 24 h) (Yankson and Moyse, 1991). After cryopreservation, the sperm suspensions were thawed for 30 and 60 s in water prepared at five different temperatures ranging from 25, 26, 27, 28 to 29 °C. Upon complete thawing, the sperm viability was immediately assessed.

4. Statistical analysis

Minitab 16 Statistical Software was used to perform all statistical analyses in this study. Sperm from lobsters of all treatments were analyzed using one-way ANOVA and significant values obtained were cross-examined using Tukey's post hoc test. Two-way ANOVA was used to evaluate the significance of sperm viability between the durations of exposure and the concentrations of cryoprotectant solution at room temperature.

5. Results

5.1. Suitable extenders

The extender, Ca-F saline, produced the highest sperm viability. Interestingly, the lowest mean sperm viability of 40.71 ± 5.19% was observed after 60 min and was similar with the rates of viability observed after 5 min exposure to the Ringer's solution and phosphate buffer (Fig. 2). These data indicated that Ringer's solution and phosphate buffer produced low mean sperm viability, as after 60 min, the observed sperm viability was only 22.06 ± 3.23% and 23.75 ± 1.49%, respectively (Fig. 2).

6. Suitable cryoprotectants.

6.1. Effects of cryoprotectant exposure on sperm viability at room temperature (25 °C)

Mean sperm viability of lobsters from the control group decreased from 72.16 ± 1.87% to 65.92 ± 5.85% after 60 min exposure at room temperature (Table 2). Moderate sperm viability was produced by 15% DMSO after 5 min of exposure (68.04 ± 0.03%), whereas 10% DMSO produced moderate-low mean sperm viability after 60 min exposure (40.29 ± 0.67%) (Table 2). In contrast, 5% EG produced the highest mean sperm viability after 5 min exposure (78.63 ± 4.96%), whereas 20% EG produced the lowest mean sperm viability after 60 min exposure (33.68 ± 5.91%) (Table 2).

The cryoprotectants, 20% glycerol, 5% methanol and 10% glycine produced the highest mean sperm viability, at 84.86 ± 3.99, 76.77 ± 7.23 and 91.87 ± 2.03%, respectively after the sperm were exposed for 5 min. However, after the sperm were exposed to 20% glycerol, 15% methanol and 20% glycine for 60 min, the mean sperm viability was significantly reduced to 10.03 ± 4.78, 55.11 ± 3.71 and 67.25 ± 1.50%, respectively (Table 2). The use of cryoprotectants appeared to significantly affect the sperm viability regardless of the concentration used (5, 10, 15 or 20%), the types of cryoprotectants used (DMSO, EG, glycerol, methanol or glycine) or the duration of exposure at room temperature (Table 2). Taken together, 10% glycine is the most suitable cryoprotectant because mean sperm viability was only slightly reduced between 5 min (91.87 ± 2.03%) and 60 min (82.25 ± 4.48%) exposure at room temperature (Table 2).

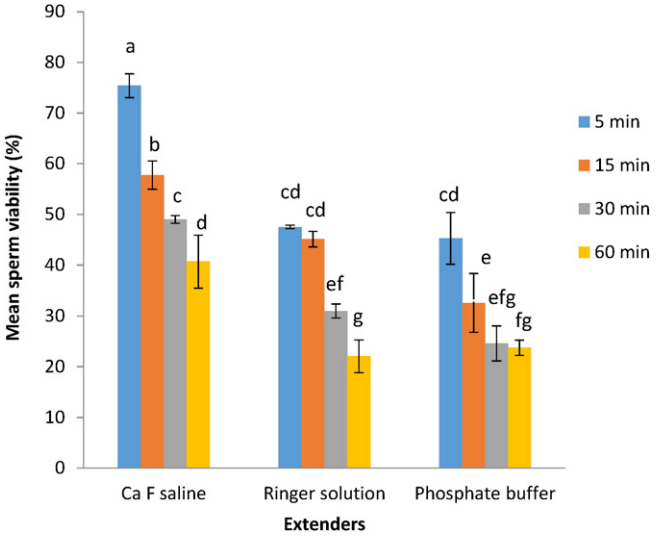

Fig. 2. The mean *P. polyphagus* sperm viability after exposure to the extenders Ca-F saline, Ringer's solution and phosphate buffer at different durations of 5, 15, 30 and 60 min. Letters indicate results that do not differ significantly from each other across treatments (combination of extender and duration of exposure) ($p < 0.05$) (mean ± S. D.).

6.2. Effects of cryoprotectant exposure on sperm viability at −4 °C

The highest mean sperm viability, at 93.68 ± 1.52, 93.53 ± 1.77 and 92.63 ± 0.51%, were achieved after exposure to 20% glycine after 6, 12 and 24 h, respectively. This was followed by exposure to 10% glycine, where mean sperm viability produced was 91.81 ± 2.33, 90.23 ± 3.51 and 84.73 ± 5.89% for the same duration of exposure. In contrast, the mean sperm viability was significantly reduced to 55.71 ± 10.58, 50.08 ± 22.39 and 34.28 ± 12.49% after exposure to 10% EG for 6, 12 and 24 h, respectively (Table 3).

6.3. Effects of cryoprotectant exposure on sperm viability at −20 °C

When 10% glycine was used as cryoprotectant, the mean sperm viability increased significantly to 91.31 ± 2.65, 91.28 ± 1.08 and 75.71 ± 8.99% after 6, 12 and 24 h exposure, respectively. However, the lowest mean sperm viability was produced in 15% EG at 29.29 ± 7.81 and 26.94 ± 3.17% after 6 and 12 h exposure, respectively (Table 4). Comparatively, when 10% methanol was used as a cryoprotectant for 24 h, the mean sperm viability produced was only 13.45 ± 1.65% (Table 4).

6.4. Effect of cryoprotectants exposure on sperm viability at −80 °C

When 10% glycine was used as a cryoprotectant for 6, 12 and 24 h, the highest mean sperm viability was produced at 75.88 ± 10.81, 72.91 ± 3.59 and 62.93 ± 9.70%, respectively. The mean sperm viability decreased moderately to 4.70 ± 2.73 and 2.83 ± 1.03% when 20% methanol was used as cryoprotectant for 6 and 12 h exposure, respectively

Table 2
Mean *P. polyhagus* sperm viability after exposure to 0, 5, 10, 15 and 20% cryoprotectants (DMSO, EG, glycerol, methanol and glycine) in different durations (5, 15, 30 and 60 min) at room temperature (25 °C). At room temperature (25 °C), 10% glycine was the best result in sperm viability. Letters indicate groups of numbers that do not significantly from each other across treatments (combination of % and type of cryoprotectant and duration of exposure) ($p < 0.05$) (mean ± S.D.).

CPAs	Time (min)			
	5 min	15 min	30 min	60 min
Control	72.16 ± 1.87^{ghijklmnop}	69.87 ± 5.69^{hijklmnopq}	68.80 ± 7.30^{jklmnopqrs}	65.92 ± 5.85^{lmnopqrstuvw}
5% DMSO	58.62 ± 0.59^{qrstuvwxyzaaabac}	53.04 ± 2.37^{yzaaabacadaeafagah}	51.7 ± 0.14^{aaabacadaeafag}	50.44 ± 3.68^{aaabacadaeafagahai}
10% DMSO	54.38 ± 4.27^{xyzaaabacadaeafag}	54.57 ± 2.45^{xyzaaabacadaeafag}	50.78 ± 1.76 ^{aaabacadaeafagahai}	40.29 ± 0.67^{aiajakalam}
15% DMSO	68.04 ± 0.03^{klmnopqrst}	66.58 ± 2.01^{lmnopqrstuv}	65.05 ± 2.43^{mnopqrstuvwxyz}	54.15 ± 0.94^{xyzaaabacadaeafagah}
20% DMSO	66.2 ± 2.17^{lmnopqrstuvw}	63.56 ± 3.61^{opqrstuvwxyz}	47.37 ± 2.22^{acadaeafagahaiaj}	47.06 ± 0.88^{adaeafagahaiajak}
5% EG	78.63 ± 4.96^{cdefghijk}	75.20 ± 0.47^{efghijklmn}	76.21 ± 0.83^{defghijklm}	63.41 ± 0.03^{opqrstuvwxyz}
10% EG	61.81 ± 0.92^{pqrstuvwxyzaa}	61.90 ± 1.51^{pqrstuvwxyzaa}	51.82 ± 1.78^{aaabacadaeafagah}	46.65 ± 6.14^{aeafagahaiajak}
15% EG	64.20 ± 0.08^{nopqrstuvwxy}	59.74 ± 5.87^{qrstuvwxyzaaab}	38.17 ± 7.45^{ajakalaman}	35.07 ± 2.28^{alamanao}
20% EG	46.29 ± 9.56^{afagahaiajakal}	35.94 ± 2.54^{akalamanao}	33.89 ± 0.55^{amanao}	33.68 ± 5.91^{amanao}
5% Glycerol	56.94 ± 15.39^{tuvwxyzaaabacadaeaf}	56.58 ± 4.50^{uvwxyzaaabacadaeaf}	43.77 ± 3.99^{agahaiajakalam}	26.09 ± 2.61^{ao}
10% Glycerol	72.80 ± 5.54^{fghijklmnop}	57.88 ± 8.32^{stuvwxyzaaabacadae}	56.45 ± 4.59^{uvwxyzaaabacadaeaf}	56.37 ± 2.40^{uvwxyzaaabacadaeaf}
15% Glycerol	58.02 ± 1.15^{rstuvwxyzaaabacad}	55.62 ± 1.11^{vwxyzaaabacadaeaf}	52.76 ± 7.68^{zaaabacadaeafagah}	42.96 ± 3.78^{ahaiajakalam}
20% Glycerol	84.86 ± 3.99^{abcde}	80.86 ± 0.84^{abcdefgh}	28.24 ± 0.64^{anao}	10.03 ± 4.78^{ap}
5% Methanol	76.77 ± 7.23^{defghijkl}	69.26 ± 0.03^{ijklmnopqr}	56.57 ± 1.98^{uvwxyzaaabacadaeaf}	56.56 ± 0.91^{uvwxyzaaabacadaeaf}
10% Methanol	73.016 ± 0.65^{fghijklmnop}	72.99 ± 1.52^{fghijklmnop}	71.32 ± 2.37^{ghijklmnop}	68.45 ± 3.57^{klmnopqrs}
15% Methanol	73.78 ± 2.48^{efghijklmno}	61.99 ± 0.22^{pqrstuvwxyzaa}	55.84 ± 1.91^{vwxyzaaabacadaeaf}	55.11 ± 3.71^{wxyzaaabacadaeaf}
20% Methanol	75.05 ± 0.86^{efghijklmn}	73.50 ± 0.90^{fghijklmno}	56.53 ± 2.31^{uvwxyzaaabacadaeaf}	56.44 ± 1.24^{uvwxyzaaabacadaeaf}
5% Glycine	89.15 ± 3.62^{abc}	89.13 ± 2.56^{abc}	87.21 ± 1.35^{abcd}	74.01 ± 0.97^{efghijklmno}
10% Glycine	**91.87 ± 2.03^{a}**	**89.28 ± 2.27^{abc}**	**87.15 ± 0.26^{abcd}**	**82.25 ± 4.48^{abcdefg}**
15% Glycine	90.39 ± 1.14^{ab}	90.14 ± 0.67^{ab}	83.93 ± 0.67^{abcdef}	80.13 ± 0.63^{bcdefghi}
20% Glycine	79.95 ± 0.76^{bcdefghij}	76.60 ± 0.31^{defghijkl}	72.97 ± 3.65^{fghijklmnop}	67.25 ± 1.50^{lmnopqrstu}

Notes: Time – Equilibrium time; CPAs – cryoprotectant types; DMSO - Dimethyl sulfoxide; EG - Ethylene glycol.

(Table 4). However, after 24 h of exposure, the mean sperm viability decreased to 2.68 ± 0.48% when 15% methanol was used (Table 5).

6.5. Cooling rate and thawing temperature

The identification of suitable cooling rates and optimum thawing temperatures was based on adaptations from Memon et al. (2012). The optimal cooling rate was achieved by Protocol I and Protocol E, as the highest mean sperm viability, at 76.09 ± 7.81 and 73.66 ± 2.16%, was achieved when the thawing temperatures used were 26 and 27 °C, respectively, at a duration of 30 s (Table 6). In contrast, Protocol D produced the lowest mean sperm viability at 2.78 ± 0.21% when the thawing temperature was 26 °C at a duration of 60 s (Table 7). There were significant differences for mean sperm viability at different thawing temperatures (25, −4, −20 and −80 °C) and durations (30 and 60 s) ($p < 0.05$).

7. Discussion

Through the selection of a suitable extender, cryoprotectant, cooling rate and thawing temperature, *P. polyphagus* sperm was able to survive the cryopreservation process in liquid nitrogen (−196 °C). However, the concentration of cryoprotectants, exposure duration, and cooling and thawing temperatures inversely affect the sperm viability.

Ca-F saline is the most suitable extender for the cryopreservation of *P. polyphagus* sperm, as it produced the highest mean sperm viability. The adapted protocol used was obtained from cryopreservation techniques to preserve spermatophores of the Banana shrimp, *P. merguiensis* (Memon et al., 2012) and the Giant tiger prawn, *P. monodon* (Vuthiphandchai et al., 2007). In the cryopreservation protocol for the preservation of the spermatophores and seminal plasma of the Mud crab, *S. serrata*, phosphate buffer was used as the extender, and glycerol, DMSO and trehalose were used as cryoprotectants (Jeyalectumic and Subramoniam, 1989). Using the same extender,

Table 3
Mean *P. polyphagus* sperm viability after exposure to different cryoprotectant types (DMSO, EG, glycerol, methanol and glycine), concentrations (0, 5, 10, 15 and 20%) and durations (6, 12 and 24 h) at −4 °C. At 4 °C, 20% glycine was the best result in sperm viability. Letters indicate groups of numbers that do not significantly from each other across treatments (combination of % and type of cryoprotectant and duration of exposure) ($p < 0.05$) (mean ± S.D.).

Time (h)	CPAs	Cryoprotectant concentration (%)				
		0% (Control)	5%	10%	15%	20%
6 h	DMSO	43.17 ± 3.73^{mno}	60.89 ± 9.44^{fghijklmno}	71.03 ± 10.43^{abcdefghijkl}	75.77 ± 10.97^{abcdefghijkl}	63.34 ± 6.42^{efghijklmn}
	EG		67.11 ± 10.16^{abcdefghijklmn}	55.71 ± 10.58^{ijklmno}	57.75 ± 22.10^{ghijklmno}	56.91 ± 10.24^{ghijklmno}
	Glycerol		73.90 ± 3.67^{abcdefghijkl}	82.74 ± 5.03 ^{abcdefgh}	82.38 ± 9.89^{abcdefgh}	88.69 ± 4.21^{abcde}
	Methanol		56.63 ± 12.31^{hijklmno}	70.10 ± 3.22^{abcdefghijkl}	73.38 ± 4.96^{abcdefghijkl}	65.02 ± 3.58^{defghijklmn}
	Glycine		75.42 ± 7.86^{abcdefghijkl}	91.81 ± 2.33^{abc}	91.04 ± 4.92^{abcd}	**93.68 ± 1.52^{a}**
12 h	DMSO	41.83 ± 5.20^{no}	54.40 ± 7.42^{jklmno}	67.63 ± 12.28^{abcdefghijklmn}	66.07 ± 7.60^{bcdefghijklmn}	63.27 ± 6.31^{efghijklmn}
	EG		63.21 ± 15.59^{efghijklmn}	50.08 ± 22.39^{lmno}	57.44 ± 11.37^{ghijklmno}	51.24 ± 10.30^{lmno}
	Glycerol		73.34 ± 11.60^{abcdefghijkl}	81.05 ± 6.63^{abcdefghi}	79.99 ± 4.74^{abcdefghijk}	83.42 ± 3.91^{abcdefg}
	Methanol		56.47 ± 8.26^{hijklmno}	68.09 ± 4.18^{abcdefghijklmn}	51.13 ± 6.61^{lmno}	62.87 ± 13.58^{efghijklmn}
	Glycine		75.13 ± 14.76^{abcdefghijkl}	90.23 ± 3.51^{abcd}	92.11 ± 2.72^{abc}	**93.53 ± 1.77^{a}**
24 h	DMSO	41.69 ± 3.88^{no}	54.25 ± 7.78^{jklmno}	67.17 ± 5.96^{abcdefghijklmn}	65.69 ± 9.46^{cdefghijklmn}	63.18 ± 9.51^{efghijklmn}
	EG		62.06 ± 4.69^{fghijklmn}	34.28 ± 12.79^{o}	56.67 ± 14.14^{hijklmno}	51.07 ± 7.05^{lmno}
	Glycerol		71.74 ± 3.38^{abcdefghijkl}	80.82 ± 12.49^{abcdefghij}	69.46 ± 4.03^{abcdefghijklm}	83.29 ± 3.39^{abcdefg}
	Methanol		55.20 ± 8.35^{ijklmno}	51.63 ± 10.35^{lmno}	42.03 ± 10.35^{no}	53.67 ± 5.25^{klmno}
	Glycine		49.84 ± 11.40^{lmno}	84.73 ± 5.89^{abcdef}	89.26 ± 4.32^{abcde}	**92.63 ± 0.51^{ab}**

Notes: Time – Equilibrium time; CPAs – cryoprotectant types; DMSO - Dimethyl sulfoxide; EG - Ethylene glycol.

Table 4
Mean *P. polyphagus* sperm viability after exposure to different cryoprotectant types (DMSO, EG, glycerol, methanol and glycine), concentrations (0, 5, 10, 15 and 20%) and durations (6, 12 and 24 h) at −20 °C. At 20 °C, 10% glycine was the best result in sperm viability. Letters indicate groups of numbers that do not significantly from each other across treatments (combination of % and type of cryoprotectant and duration of exposure) ($p < 0.05$) (mean ± S.D.).

Time (h)	CPAs	Cryoprotectant concentration (%)				
		0% (Control)	5%	10%	15%	20%
6 h	DMSO	$48.82 \pm 10.46^{jklmnopq}$	$62.23 \pm 12.87^{efghijklm}$	$67.79 \pm 2.80^{bcdefghijk}$	$64.92 \pm 3.07^{efghijkl}$	$68.94 \pm 4.40^{abcdefghij}$
	EG		$74.06 \pm 7.64^{abcdefghi}$	$70.00 \pm 7.79^{abcdefghij}$	29.29 ± 7.81^{qrs}	30.07 ± 4.50^{qrs}
	Glycerol		$54.07 \pm 16.28^{ijklmnop}$	$70.66 \pm 4.57^{abcdefghij}$	$69.13 \pm 3.22^{abcdefghij}$	$63.43 \pm 7.55^{efghijkl}$
	Methanol		81.65 ± 3.78^{abcdefg}	34.97 ± 4.93^{pqrs}	36.45 ± 10.43^{opqrs}	$47.41 \pm 11.44^{jklmnopq}$
	Glycine		82.54 ± 5.21^{abcd}	**91.31 ± 2.65^{a}**	90.17 ± 3.83^{abc}	90.17 ± 4.24^{abc}
12 h	DMSO	39.75 ± 4.35^{mnopqr}	$61.46 \pm 11.97^{efghijklmn}$	$65.59 \pm 7.36^{defghijkl}$	$57.34 \pm 10.50^{hijklmnop}$	$59.13 \pm 11.24^{efghijklmno}$
	EG		$74.05 \pm 5.60^{abcdefghi}$	$68.38 \pm 7.62^{abcdefghij}$	26.94 ± 3.17^{qrs}	28.01 ± 14.65^{qrs}
	Glycerol		49.79 ± 9.08^{jklmnop}	$60.14 \pm 8.83^{efghijklmn}$	$63.48 \pm 2.46^{efghijkl}$	$63.24 \pm 9.83^{efghijkl}$
	Methanol		$79.33 \pm 6.48^{abcdefgh}$	34.94 ± 7.16^{pqrs}	36.38 ± 11.05^{opqrs}	$43.21 \pm 21.08^{lmnopqr}$
	Glycine		82.35 ± 3.26^{abcdef}	**91.28 ± 1.08^{a}**	90.83 ± 5.35^{abc}	91.04 ± 1.77^{ab}
24 h	DMSO	38.75 ± 4.85^{nopqr}	$59.07 \pm 8.36^{fghijklmno}$	$63.74 \pm 12.78^{efghijkl}$	$54.74 \pm 3.30^{ijklmnop}$	$58.68 \pm 5.62^{ghijklmno}$
	EG		$57.34 \pm 2.02^{hijklmnop}$	$67.51 \pm 3.17^{cdefghijk}$	26.36 ± 4.89^{qrs}	27.89 ± 6.20^{qrs}
	Glycerol		$44.87 \pm 5.73^{klmnopqr}$	$56.11 \pm 12.96^{hijklmnop}$	$63.32 \pm 7.15^{efghijkl}$	$56.61 \pm 10.06^{hijklmnop}$
	Methanol		$65.75 \pm 8.37^{defghijkl}$	13.45 ± 1.65^{s}	23.47 ± 8.09^{rs}	22.06 ± 8.09^{rs}
	Glycine		81.73 ± 7.58^{abcdefg}	**$75.71 \pm 8.99^{abcdefghi}$**	88.83 ± 4.16^{abcd}	89.04 ± 3.08^{abcd}

Notes: Time – Equilibrium time; CPAs – cryoprotectant types; DMSO - Dimethyl sulfoxide; EG - Ethylene glycol.

another cryopreservation technique for the sperm of the Edible rock lobster, *Panulirus homarus* employed glycerol, DMSO, methanol and glucose as cryoprotectants (Sasikala and Meena, 2009). Taken together, these findings demonstrate that the efficiency of cryoprotectants and the durations of their exposure to gametes vary from species to species.

There are the following two types of cryoprotectants: intracellular cryoprotectants (with low molecular weights; e.g., DMSO, glycerol, and glycine.) and extracellular cryoprotectants (with high molecular weights that do not penetrate cells; e.g., sucrose). In this study, the cryoprotectants (e.g., DMSO, EG, glycerol, methanol and glycine) were used at concentrations of 5, 10, 15 and 20% to cryopreserve the sperm of *P. polyphagus*. Comparatively, Memon et al. (2012) reported the use of DMSO, EG, methanol, glycerol, sucrose and magnesium chloride as cryoprotectants for *P. merguiensis*. In addition, DMSO and glycerol are common cryoprotectants used for the cryopreservation of crustacean sperm (Chow et al., 1985; Anchordoguy et al., 1988; Bhavanishankar and Subramoniam, 1997). Hence, the selection of cryoprotectants to improve gamete viability after the cryopreservation process depends on the species.

DMSO is generally used for oyster and marine shrimp gamete cryopreservation (Hughes, 1973; Anchordoguy et al., 1988; Yankson and Moyse, 1991). However, glycerol was instead used for gamete cryopreservation of the horseshoe crab, *Limulus polyphemus* (Behlmer and Brown, 1984), and the crab (Bhavanishankar and Subramoniam, 1997; Sasikala and Meena, 2009). Moreover, the cryoprotectant solution using a combination of 5% DMSO and 10% glycerol effectively cryopreserved the sperm of *P. homarus* and the Chinese mitten crab, *Eriocheir sinensis* (Kang et al., 2009; Sasikala and Meena, 2009). In another study, two concentrations (5% and 10%) of DMSO, methanol and EG were used as cryoprotectants for the cryopreservation of *P. monodon* spermatophores (Bart et al., 2006). Upon thawing, the sperm successfully fertilized shrimp eggs via artificial insemination (Bart et al., 2006). In regard to shrimp, 10% glycerol effectively cryopreserved the spermatophores of the Giant freshwater prawn, *M. rosenbergii* (Chow et al., 1985), and the use of 15% magnesium chloride was also deemed effective for *P. merguiensis* (Memon et al., 2012). Glycine has a low molecular weight of 75.07 g mol^{-1}. Glycine permeates sperm cells and minimizes cellular damage in slow-freezing biological systems. Thus, this study uses 10% glycine as the cryoprotectant. The successful usage of glycine for the cryopreservation of sperm suspensions was also reported in *Crassostrea tulipa* and in three other species of oysters (*Saccostrea cucullata*, *C. gigas and Crassostrea iredalei*). Hence, the sperm viability of *C. gigas* increased after the addition of glycine to the cryoprotective diluents (Yankson and Moyse, 1991).

Table 5
Mean *P. polyphagus* sperm viability after exposure to different cryoprotectant types (DMSO, EG, glycerol, methanol and glycine), concentrations (0, 5, 10, 15 and 20%) and durations (6, 12 and 24 h) at −80 °C. At 80 °C, 10% glycine was the best result in sperm viability. Letters indicate groups of numbers that do not significantly from each other across treatments (combination of % and type of cryoprotectant and duration of exposure) ($p < 0.05$) (mean ± S.D.).

Time (h)	CPAs	Cryoprotectant concentration (%)				
		0% (Control)	5%	10%	15%	20%
6 h	DMSO	17.24 ± 5.84^{nopqr}	62.82 ± 6.41^{abcde}	$52.88 \pm 15.83^{abcdefghij}$	$54.41 \pm 3.63^{abcdefghi}$	$48.48 \pm 17.33^{bcdefghijkl}$
	EG		66.86 ± 5.47^{abc}	60.30 ± 7.45^{abcdef}	$39.41 \pm 11.36^{efghijklmn}$	$24.06 \pm 11.90^{klmnopqr}$
	Glycerol		61.09 ± 3.91^{abcdef}	73.15 ± 14.77^{abc}	74.98 ± 2.04^{ab}	74.63 ± 6.19^{ab}
	Methanol		20.15 ± 7.47^{mnopqr}	$28.15 \pm 9.22^{ijklmnopqr}$	8.70 ± 4.24^{opqr}	4.70 ± 2.73^{pqr}
	Glycine		67.22 ± 4.89^{abc}	**75.88 ± 10.81^{a}**	$49.34 \pm 11.71^{abcdefghijkl}$	$52.13 \pm 14.54^{abcdefghij}$
12 h	DMSO	14.73 ± 3.98^{nopqr}	$56.87 \pm 12.76^{abcdefgh}$	$50.41 \pm 9.43^{abcdefghijk}$	$53.04 \pm 9.83^{abcdefghij}$	$46.78 \pm 10.43^{cdefghijklm}$
	EG		66.85 ± 10.08^{abcd}	$52.70 \pm 12.13^{abcdefghij}$	$28.16 \pm 14.09^{ijklmnopqr}$	$23.17 \pm 8.15^{klmnopqr}$
	Glycerol		61.03 ± 5.57^{abcdef}	73.11 ± 10.58^{abc}	73.07 ± 9.05^{abc}	70.91 ± 8.11^{abc}
	Methanol		11.78 ± 3.62^{opqr}	$26.90 \pm 8.07^{jklmnopqr}$	2.91 ± 1.00^{qr}	2.83 ± 1.03^{qr}
	Glycine		67.21 ± 8.81^{abc}	**72.91 ± 3.59^{abc}**	$46.67 \pm 5.23^{cdefghijklm}$	$39.83 \pm 16.07^{defghijklmn}$
24 h	DMSO	14.68 ± 5.29^{nopqr}	58.03 ± 3.20^{abcdefg}	$50.39 \pm 7.66^{abcdefghijk}$	$35.03 \pm 14.69^{fghijklmno}$	$30.09 \pm 12.71^{hijklmnopq}$
	EG		$55.16 \pm 12.65^{abcdefghi}$	$52.50 \pm 10.82^{abcdefghij}$	$26.67 \pm 10.69^{jklmnopqr}$	22.95 ± 2.65^{lmnopqr}
	Glycerol		60.83 ± 7.01^{abcdef}	68.08 ± 5.75^{abc}	72.91 ± 9.34^{abc}	67.88 ± 2.20^{abc}
	Methanol		3.39 ± 1.15^{qr}	20.67 ± 9.61^{mnopqr}	2.68 ± 0.48^{r}	3.51 ± 1.24^{qr}
	Glycine		60.10 ± 16.81^{abcdef}	**62.93 ± 9.70^{abcde}**	$46.61 \pm 7.76^{cdefghijklm}$	$31.21 \pm 12.41^{ghijklmnop}$

Notes: Time – Equilibrium time; CPAs – cryoprotectant types; DMSO - Dimethyl sulfoxide; EG - Ethylene glycol.

Table 6
Mean sperm viability at different cooling rates protocols and thawing temperatures at 25–29 °C (30 s) for *P. polyphagus* sperm cryopreservation. The cooling rates of Protocol I were the best result for *P. polyphagus* sperm (thawing temperature for 26 °C and duration for 30 s). Letters indicate groups of numbers that do not differ significantly from each other across treatments (combination of cooling rate protocols and thawing temperatures) ($p < 0.05$) (mean ± S.D.).

Cooling rates protocols	Thawing temperature				
	25 °C, 30 s	26 °C, 30 s	27 °C, 30 s	28 °C, 30 s	29 °C,30 s
A	0^{j}	0^{j}	6.02 ± 0.03^{hij}	6.84 ± 3.34^{hij}	0^{j}
B	0^{j}	29.39 ± 0.80^{b}	11.31 ± 0.58^{efghi}	16.26 ± 1.87^{cdef}	0^{j}
C	13.14 ± 0.87^{defgh}	0^{j}	8.76 ± 0.41^{fghi}	15.77 ± 1.83^{cdefg}	0^{j}
D	0^{j}	0^{j}	0^{j}	0^{j}	0^{j}
E	0^{j}	69.46 ± 3.99^{a}	73.66 ± 2.16^{a}	0^{j}	0^{j}
F	9.80 ± 4.24^{fghi}	0^{j}	4.06 ± 0.04^{ij}	9.52 ± 0.48^{fghi}	0^{j}
G	0^{j}	19.24 ± 0.25^{cd}	0^{j}	7.95 ± 0.04^{ghi}	0^{j}
H	0^{j}	0^{j}	6.87 ± 0.16^{hij}	0^{j}	0^{j}
I	6.77 ± 0.42^{hij}	**76.09 ± 7.81^{a}**	16.44 ± 0.53^{cdef}	0^{j}	9.77 ± 0.29^{fghi}
J	15.68 ± 0.09^{cdefg}	0^{j}	3.56 ± 0.34^{ij}	0^{j}	0^{j}
K	6.35 ± 0.02^{hij}	21.49 ± 7.14^{c}	0^{j}	0^{j}	0^{j}
L	0^{j}	17.84 ± 5.11^{cde}	13.14 ± 4.92^{defgh}	0^{j}	0^{j}

At room temperature, the percentage of mean sperm viability increased when 10% glycine was used 91.87 ± 2.03% (5 min) compared to the control treatment (72.16 ± 1.87% (5 min)) and to other cryoprotectants in this study. The lowest mean sperm viability produced was 46.29 ± 9.56% (5 min) at room temperature was achieved with 20% EG. For exposure to −4 °C (for 6, 12 and 24 h), 20% glycine produced higher mean sperm viability than 10% glycine. Moreover, for exposure to −20 and −80 °C, 10% glycine produced the highest percentage of mean sperm viability, at 91.31 ± 2.65, 91.28 ± 1.08 and 75.71 ± 8.99% (6, 12 and 24 h at −20 °C) and 75.88 ± 10.81, 72.91 ± 3.59 and 62.93 ± 9.70% (6, 12 and 24 h at −80 °C). Thus, all of the room temperature data indicate that for exposures to −4, −20 and −80 °C, 10% glycine is optimal and was thus selected to cryopreserve *P. polyphagus* sperm in liquid nitrogen (−196 °C).

Thawing temperatures and durations indefinitely affected the viability of *P. polyphagus* sperm. In this study, thawing temperatures of cryopreserved lobster sperm ranged between 25 and 29 °C at durations of 30 and 60 s. The adapted study of Memon et al. (2012) selected thawing temperatures of 25, 27, 29, 31 and 33 °C, and thawing durations lasted between 60, 120, 180, 240 and 300 s. In comparison to this study, the selection of thawing temperatures and durations by Memon et al. (2012) were higher because spermatophores were tested instead of sperm. Spermatophores not only store and relocate the sperm of male decapod crustaceans but also aid the relocation of sperm to the receptacle of females (Sasikala and Subramoniam, 1987; Hinsch, 1991).

The process of cryopreserve thawing is either slow or rapid and applies to sperm, spermatophores, larvae, and embryos. The cooling rates of Protocol I were most suitable for *P. polyphagus* sperm. In this case, the thawing temperature of 26 °C and the duration for 30 s produced the highest sperm viability at 76.09 ± 7.81% after 24 h cryopreservation in liquid nitrogen. Thawing of sperm at temperatures between 15 and 30 °C is generally conducted in a water bath (Dunn and McLachlan, 1973; Hughes, 1973; Hwang and Chen, 1973; Staeger, 1974; Asahina and Takahashi, 1978; Bougrier and Rabenomanana, 1986; Iwata et al., 1989; Kurokura et al., 1989, 1990; Bury and Olive, 1993; Tsai and Chao, 1994; McFadzen, 1995). For some organisms, gamete fertility is enhanced upon rapid thawing (Gwo, 2000) or at higher temperatures (55–60 °C) (Yankson and Moyse, 1991; Zell et al., 1979). Based on the protocol described by Memon et al. (2012), cryopreserved spermatophores of *P. merguiensis* that were thawed at 27 °C for 120 s exhibited increased sperm viability.

Slow-freezing rates generate deleterious extracellular ice crystals in gametes (Woods et al., 2004), and re-crystallization of water in gametes from this slow process also results in cellular damage (Farrant et al., 1977a, 1977b). Comparatively, fast-freezing rates reduce intracellular ice formation during the cryopreservation process (Woods et al., 2004). The gametes of the White leg shrimp, *L.vannamei* (Lezcano et al., 2004), *S. serrata* (Jeyalectumic and Subramoniam, 1989; Billard et al., 1995), *S. ingentis* (Anchordoguy et al., 1988), *L. polyphemus* (Behlmer and Brown, 1984) and *M. rosenbergii* (Chow et al., 1985; Akarasanon et al., 2004) exhibited high sperm viability despite being subjected to slow-freezing. Hence, the selection of cooling rates for cryopreservation is greatly species-dependent (Suquet et al., 2000; Gwo, 2000). This study found that the cryopreservation of *P. polyphagus* sperm was optimized with slow-cooling rates and fast thawing temperatures. Indeed, slow-cooling rates reduced intracellular

Table 7
Mean sperm viability at different cooling rates protocols and thawing temperatures at 25–29 °C (60 s) for *P. polyphagus* sperm cryopreservation. The cooling rates of Protocol C were the best result for *P. polyphagus* sperm (thawing temperature for 26 °C and duration for 60 s). Letters indicate groups of numbers that do not differ significantly from each other across treatments (combination of cooling rate protocols and thawing temperatures) ($p < 0.05$) (mean ± S.D.).

Cooling rates protocols	Thawing temperature				
	25 °C, 60 s	26 °C, 60 s	27 °C, 60 s	28 °C, 60 s	29 °C, 60 s
A	0^{m}	7.24 ± 0.53^{hijklm}	16.47 ± 5.20^{efghij}	0^{m}	$13.67 \pm 1.12^{efghijkl}$
B	0^{m}	0^{m}	13.08 ± 4.68^{fghijkl}	19.12 ± 7.18^{bcdefg}	0^{m}
C	0^{m}	**34.57 ± 0.55^{a}**	0^{m}	0^{m}	0^{m}
D	8.26 ± 0.34^{ghijklm}	2.78 ± 0.21^{lm}	8.54 ± 0.15^{ghijklm}	0^{m}	0^{m}
E	5.39 ± 0.12^{jklm}	0^{m}	19.28 ± 4.99^{bcdefg}	0^{m}	0^{m}
F	11.47 ± 3.04^{fghijkl}	0^{m}	15.76 ± 4.12^{efghijk}	9.77 ± 1.62^{fghijklm}	$10.24 \pm 1.72^{fghijklm}$
G	6.69 ± 0.41^{ijklm}	18.56 ± 6.85^{cdefgh}	17.99 ± 10.79^{defghi}	0^{m}	$10.20 \pm 0.11^{fghijklm}$
H	0^{m}	0^{m}	20.31 ± 3.56^{bcdef}	0^{m}	0^{m}
I	12.68 ± 1.22^{fghijkl}	27.97 ± 0.46^{abcd}	29.92 ± 5.96^{ab}	0^{m}	15.94 ± 0.06^{efghij}
J	0^{m}	29.32 ± 6.52^{abc}	11.92 ± 0.66^{fghijkl}	0^{m}	0^{m}
K	9.13 ± 0.13^{fghijklm}	6.62 ± 0.49^{jklm}	24.46 ± 12.73^{abcde}	0^{m}	0^{m}
L	3.93 ± 1.86^{lm}	4.56 ± 0.43^{klm}	31.89 ± 17.58^{a}	0^{m}	0^{m}

ice formation and maintained the osmotic balance of the sperm. Fast-thawing temperatures reduced biochemical leakage, increasing sperm cell survival (Simione, 2009). From all of the results obtained from this study, Protocol I is optimal because it produced the highest viability compared to the other protocols tested.

8. Conclusion

The optimal protocol found by this study is Protocol I, which included Ca-F saline as an extender; 10% glycine as a cryoprotectant; a cooling rate of 15 min; cooling temperatures of 25, 20, 16, 4, 2, −4, −20, −80, −110, −150 and −196 °C; and a thawing temperature of 26 °C for 30 s. This protocol has the optimal conditions and produced high sperm viability for the cryopreservation of sperm from *P. polyphagus*.

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