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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).

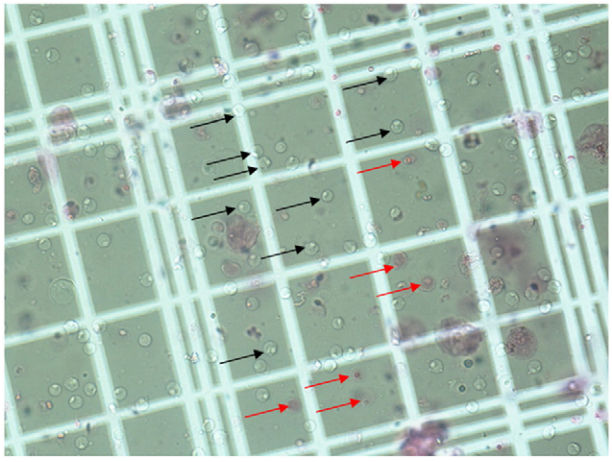

Fig. 1. Black arrows indicate live unstained sperm (viable sperm) cells, whereas the red arrows indicate dead sperm cells of *P. polyphagus*. The sperm cells were observed under 40× magnification.