

Cryopreservation of spermatogonia of the giant freshwater prawn, *Macrobrachium rosenbergii*, using slow and ultra-rapid freezing

Tomoyuki Okutsu^{a,*}, Natthida Rakbanjong^b, Shinya Shikina^{c,d}, Misako Miwa^e,
Monwadee Wonglapsuwan^{b,f,**,1}

^a Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki, Japan

^b Division of Biological Science, Faculty of Science, Prince of Songkla University, Hatyai, Songkhla, Thailand

^c Institute of Marine Environment and Ecology, National Taiwan Ocean University, Keelung, Taiwan

^d Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan

^e Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo, Japan

^f Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hatyai, Songkhla, Thailand

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ABSTRACT

The giant freshwater prawn, *Macrobrachium rosenbergii*, is an important species that is widely raised in tropical and subtropical regions. To develop techniques for preserving valuable traits in this species, we have devised protocols for cryopreserving spermatogonia—progenitor cells that give rise to spermatozoa through spermatogenesis—using slow freezing and ultra-rapid freezing techniques. Optimization of cryoprotectants and their concentrations revealed that 10 % dimethyl sulfoxide (ME₂SO) is effective as a cryoprotectant in both methods. The optimal time for equilibration to the cryoprotectant before slow freezing was determined to be 15 min. Thawing temperature had a significant effect on cell recovery and viability, both being markedly higher when cells were thawed at 10 °C than at 27 °C. After long-term storage in liquid nitrogen, cells preserved by ultra-rapid freezing exhibited higher recovery and viability rates than those preserved using the slow freezing method, with recovery rates of up to 86.4 % ± 5.9 % at 6 months. Therefore, using 10 % ME₂SO with the ultra-rapid freezing method is recommended as the optimal protocol for long-term cryopreservation of *M. rosenbergii* spermatogonia.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, is one of the most important freshwater prawn species cultured in tropical and subtropical regions [30]. In 2021, global production of this species reached 313,756 t, valued at over 2.45 × 10⁹ USD [37], and production has been steadily increasing worldwide (FAO FishStat 2024: <https://www.fao.org/fishery/statistics-query/en/aquaculture>). There is growing interest in the basic biology and pathology of this species [3,14,47]. Continuing systematic stock improvement programs for this species, aimed at enhancing economically important traits [49], holds the potential to further boost productivity.

The development of techniques not only to increase production but also to preserve valuable traits is a priority in aquaculture. In recent

years, new aquaculture technologies based on germ cell cryopreservation and transplantation techniques have been established for fish [57]. By transplanting thawed cryopreserved germ cells into an allogeneic or closely related heterologous recipient, it is possible for such recipient to produce the next generation derived from previously frozen cells [56]. This technology can minimize the risk of loss of valuable strains to disease or unexpected accidents. The use, as recipients, of closely related species with smaller body sizes and shorter generation times also allows for faster and more efficient seed production in a smaller space [57]. To apply this technology to aquacultured species other than fish, studies have centered on marine shrimp species, including *Penaeus monodon*, *Fenneropenaeus merguensis*, and *Litopenaeus vannamei* [4,40]. However, no cryopreservation or transplantation studies of any freshwater shrimp species have so far been reported.

* Corresponding author.

** Corresponding author. Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.

E-mail addresses: okutsut0312@jircas.go.jp (T. Okutsu), natthida.rbj@gmail.com (N. Rakbanjong), shikina@mail.ntou.edu.tw (S. Shikina), mmiwa00@kaiyodai.ac.jp (M. Miwa), monwadee.wo@gmail.com (M. Wonglapsuwan).

¹ These two authors share senior authorship.

Cryopreservation uses low temperatures to preserve living cells or tissues [35]. Slow cooling combined with the use of cryoprotectants is commonly used to protect cells from damage caused by crystalline ice formation [10]. However, slow freezing can still cause some cell damage owing to the presence of ice crystals. To address this issue, rapid freezing methods, such as vitrification, have been developed [15,54]. Vitrification involves rapid cooling to temperatures below the cryoprotectant's glass transition point, thus preventing ice crystal formation [21,54]. The success of cryopreservation depends on several factors, including the type and concentration of the cryoprotectant, rates of cooling and thawing, and equilibration time [54]. Since cells vary as to water permeability, tolerance to toxicity, lipid content, and size, the conditions for cryopreservation must be optimized for each cell type and species.

As a first step in advancing aquaculture technology for *M. rosenbergii*, we devised protocols for cryopreserving spermatogonia—the progenitor cells that give rise to spermatozoa through spermatogenesis—using both slow freezing and ultra-rapid freezing methods, with the latter being a technique that incorporates aspects of vitrification, particularly in its rapid temperature reduction, as investigated in this study. We first identified spermatogonia within dispersed testicular cells by the use of a germ-cell molecular marker and determined their morphological characteristics and size. We then optimized the type and concentration of cryoprotectant, equilibration time, and thawing temperature for effective cryopreservation.

2. Materials and methods

2.1. Shrimp

Fifty wild giant freshwater prawns (*M. rosenbergii*) were purchased from local fishermen in October 2019 and another fifty in January 2020, and were stocked in the laboratory until use. The fishermen collected the prawns from the wild population of Songkhla Lake, located in Songkhla Province, Thailand. From the individuals stocked, 4–5 prawns were randomly selected and used for each experiment. They had a mean body weight of 36.2 ± 8.8 g and a mean body length of 16.0 ± 1.4 cm. The gonad-somatic index (GSI) of the individuals was $0.27\% \pm 0.12$ (mean \pm SD), ranging from 0.09 % to 0.54 %. They were maintained in an aerated concrete pond (1.0 m \times 2.0 m \times 0.8 m) at 27–28 °C for 5–7 days before experimentation. They were fed twice a day with commercial feed (Krunghthai Food Public Co., Ltd., Thailand). Half of the culture water was changed daily. All the experiments were carried out in accordance with the *Guidelines for the Care and Use of Laboratory Animals*, Prince of Songkhla University, Songkhla, Thailand.

2.2. Testis collection and testicular cell preparation

Testes were isolated under a stereomicroscope and cut into small pieces weighing approx. 18–20 mg. The pieces prepared from one shrimp were divided into several groups and used in both the experimental and control groups. One shrimp was used as one replicate in the analysis. Before experimentation, the testis pieces were maintained in Leibovitz's L-15 medium (Gibco, MA, USA) supplemented with D-glucose (Thermo Fisher Scientific, MA, USA) at a final concentration of 27.75 mM, fetal bovine serum (FBS; Gibco, MA, USA) at 10 %, and Antibiotic-Antimycotic (15240096, Gibco-Thermo Fisher Scientific, MA, USA) at 1 %. The pH of the supplemented L-15 medium was adjusted to pH 7.4. To obtain testicular cell suspensions, the testis pieces were enzymatically dissociated in supplemented L-15 medium containing 2.0 mg/mL of collagenase H (LS004176, Worthington Biochemical Corp., USA), 150 U/mL of deoxyribonuclease I (2929385, EMD Millipore Corp., USA), and 1.65 mg/mL of Dispase II (D4693-1G, Sigma-Aldrich, USA) for a total of 1 h [17]. Gentle pipetting was applied at 0, 30, and 60 min during the 1-h incubation period. The enzymatic reactions were terminated by adding excess supplemented L-15 medium. The resulting cell suspensions were filtered through a nylon screen with a pore size of 42 μ m to

eliminate retained cell clumps and then stored on ice until use.

2.3. Immunohistochemistry

The isolated testes were fixed in Bouin's fixative at 4 °C with gentle shaking for 7 h, then transferred into 70 % ethanol and held at 4 °C until use. To identify spermatogonia in the testes, we performed immunohistochemical analysis according to Ichida et al. [18] with some modifications. In brief, the fixed testes were dehydrated, embedded in paraffin, and sectioned into 5- μ m slices. The sections were deparaffinized and rehydrated through a xylene and ethanol series. Antigen retrieval treatment was then conducted using Histo VT One solution (Nacalai Tesque, Kyoto, Japan) at 90 °C for 20 min, followed by blocking in Block-Ace (DS Pharma Biomedical, Osaka, Japan) at room temperature for 30 min. The sections were then incubated overnight at 4 °C with 1:2000-diluted anti-zebrafish vasa antibody (ab209710: Abcam, Cambridge, UK) in Can Get Signal™ immunostain solution A (Toyobo, Osaka, Japan). This dilution of primary antibody was determined as optimal based on preliminary testing of various concentrations (1:100, 1:500, 1:1000, 1:2000, 1:3000, and 1:5000), with 1:2000 yielding the clearest and most reliable results. They were then incubated for 1 h at room temperature with 1:200-diluted Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, MA, US) in Can Get Signal™ immunostain solution B (Toyobo). The concentration of the secondary antibody used was selected based on the methodology described by Ichida et al. [17], as this dilution provided optimal results in the present experiments. Images were captured using a fluorescence microscope (IX70, Olympus, Tokyo, Japan), and germ cell stages were identified based on their morphological characteristics as described in previous studies [33,38].

2.4. Immunocytochemistry

The dissociated testicular cells were subjected to immunocytochemistry. A cell suspension ($2 - 5 \times 10^4$ cell/mL) was fixed in 4 % paraformaldehyde (PFA) diluted in phosphate buffer saline (PBS) for 5 min. One drop of cell suspension was placed onto an MAS-coated glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan) and dried in a laminar flow cabinet for 4–6 h at room temperature. The cells were then immunocytochemically stained with the above-described commercially available anti-zebrafish vasa antibody. Cell images were captured through a fluorescence microscope (IX70, Olympus), and the cell diameter was measured using cellSens Standard imaging software (Olympus). The cells were categorized into three groups by diameter: <8 μ m, 8–10 μ m, and >10 μ m. The percentage of immunoreactivity-positive cells in each group was then determined as (immunoreactivity-positive cells/total cells) \times 100. More than 100 cells were counted in each group. Four replicates using four specimens each were used.

2.5. Western blotting

Total protein from shrimp testis was extracted using the TRIzol™ reagent following the manufacturer's protocol. Briefly, shrimp testes were dissected, and 1 mL of TRIzol™ reagent was added to 50 mg of testis tissue. The tissue was homogenized using a homogenizer, and the homogenate was centrifuged at $12,000 \times g$ for 10 min at 4 °C. The supernatant was transferred to a new tube and incubated at 25 °C for 5 min. Next, 0.2 mL of chloroform was added, and the mixture was vigorously shaken. The tube was incubated at 25 °C for 3 min and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The interphase was collected, and 0.3 mL of 100 % ethanol was added. The sample was gently mixed by inversion, incubated at 25 °C for 3 min, and centrifuged at $2000 \times g$ for 10 min at 4 °C to pellet the DNA. The supernatant was then transferred, and proteins were precipitated by adding 1.5 mL of isopropanol. This mixture was centrifuged at $12,000 \times g$ for 10 min at 4 °C to pellet the proteins. The protein pellet was washed twice with 0.3 M guanidine hydrochloride and centrifuged at $7500 \times g$ for 5 min at 4 °C.

It was further washed with 2 mL of 100 % ethanol, briefly vortexed, and centrifuged again at $7500\times g$ for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 200 μ L of 1 % SDS. To remove insoluble materials, the sample was centrifuged at $10,000\times g$ for 10 min at 4 °C. The supernatant containing soluble proteins was transferred to a new tube. Protein concentration was measured using the Lowry assay [28]. For SDS-PAGE, 5, 10, and 20 μ g of total protein were loaded onto a 12 % SDS-PAGE gel and separated by electrophoresis. The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Germany) using a semi-dry transfer system at 150 mA for 1 h. The membranes were blocked with 5 % skim milk in phosphate-buffered saline with 0.1 % Tween 20 (PBST) at room temperature overnight. The next day, the membranes were incubated overnight at 4 °C with the anti-zebrafish vasa antibody (ab209710: Abcam, Cambridge, UK) as the primary antibody at a 1:2000 dilution. After washing three times with PBST (5 min each), the membranes were incubated with HRP-conjugated anti-Rabbit IgG secondary antibody (1:2000, Cell Signal, cat no. 7074S) for 2 h at room temperature. The membranes were washed three more times with PBST (5 min each). Protein bands were visualized using SuperKine West Pico Plus Chemiluminescent Substrate (Abbkine, USA) and imaged with a Chemiluminescence Fusion SOLO S system (Vilber, Germany). Precision Plus Protein Standard (Bio-Rad, Germany) was used as a molecular weight marker.

2.6. Cell recovery and viability assessment

Fresh or frozen-thawed testes were enzymatically dissociated as above. The cells were then stained with trypan blue (Fig. 4A) and counted using a hemacytometer under a light microscope (BX53, Olympus, Tokyo, Japan). From the results of immunohistochemical and immunocytochemical analyses, we regarded 8 - 10 μ m-diameter cells as spermatogonia. Percentage viability was calculated as (number of trypan blue-negative spermatogonia/total number of [trypan blue-positive + trypan blue-negative] spermatogonia) \times 100. Taking into account losses caused by manipulation (e.g., pipetting) and cell-burst during the experiment, we determined the percentage recovery efficiency as (number of trypan blue-negative spermatogonia in 1 mg of frozen-thawed testis/number of trypan blue-negative spermatogonia in 1 mg of fresh testis) \times 100. Five replicates using five specimens each were used in each test.

2.7. Slow freezing

The isolated testes were slow-frozen according to Lee et al. [26] with some modifications. In brief, the testes were isolated and cut into small pieces as above. After the pieces had been weighed, one piece was used as a freshly prepared control and the remainder were used as the test group. The pieces were transferred into 1.8-mL cryovials with 500 μ L cryomedium containing 0.1 M trehalose, 15 % bovine serum albumin, and extender as cryoprotectant. The extender was composed of 19.46

mM HEPES, 132.17 mM NaCl, 2.56 mM KCl, 8.13 mM KH_2PO_4 , 1.34 mM Na_2HPO_4 , 1.28 mM sodium pyruvate, 0.92 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, and 0.49 mM $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, at pH 7.8. The testis pieces were incubated on ice for 15 min and cooled at 1 °C/min for 90 min in a Bicell freezing container (Nihon Freezer Co., Ltd., Tokyo, Japan) held at -80 °C before being plunged into liquid nitrogen (-196 °C) for storage. The pieces were later thawed at 10 °C in a water bath for 1 min. They were transferred to the supplemented L-15 medium and dissociated. The viability and recovery of the spermatogonia were subsequently examined as described above. Freshly prepared testis pieces were used as the control. Five replicates using five specimens each were used in each test.

2.8. Ultra-rapid freezing

Ultra-rapid freezing of testes was performed following the method outlined by Seki et al. [44]. In brief, as illustrated schematically in Fig. 1, a testis piece was placed on a copper mesh with a pore size of 40 μ m and transferred into 24-well plate with 1 mL ultra-rapid freezing medium which contained, as cryoprotectants, 0.2 M trehalose, 15 % ethylene glycol, 20 % FBS, and extender with slightly different components from those used for slow freezing: 26.8 mM HEPES, 182.1 mM NaCl, 3.5 mM KCl, 11.2 mM KH_2PO_4 , 1.5 mM Na_2HPO_4 , 1.75 mM sodium pyruvate, 0.7 mM $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, and 1.3 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, at pH 7.8. The testis pieces were incubated for 30 min in ultra-rapid freezing medium on ice. The piece on the copper mesh was then transferred into a 1.8-mL cryovial containing liquid nitrogen and rapidly plunged into the liquid nitrogen tank. After a week of storage in liquid nitrogen, the piece on the copper mesh was thawed by direct immersion in L-15 medium containing 10 % FBS with shaking for 5 min, with three changes of medium. The testis pieces were dissociated and the viability and recovery of the spermatogonia were investigated as described above. Freshly prepared testis pieces were used as the control. Five replicates using five specimens each were used in each test.

2.9. Optimization of cryoprotectants

To investigate the effect of each cryoprotectant on cell viability and recovery, we tested 10 % ME_2SO , 10 % glycerol, or 10 % MgCl_2 in both slow freezing and ultra-rapid freezing methods. After storage in liquid nitrogen for a week, the slow-frozen testis pieces were thawed in a water bath at 10 °C, while the ultra-rapidly frozen testis pieces were thawed by direct immersion in L-15 medium containing 10 % FBS at 10 °C. The testicular cells were then prepared, and the viability and recovery of spermatogonia were evaluated as described above. Freshly prepared testis pieces were used as the control. Five replicates using five specimens each were used for each set of conditions.

2.10. Optimization of cryoprotectant concentration

To optimize the concentration of cryoprotectant, we used 5 %, 10 %, 15 %, and 20 % concentrations of each cryoprotectant.

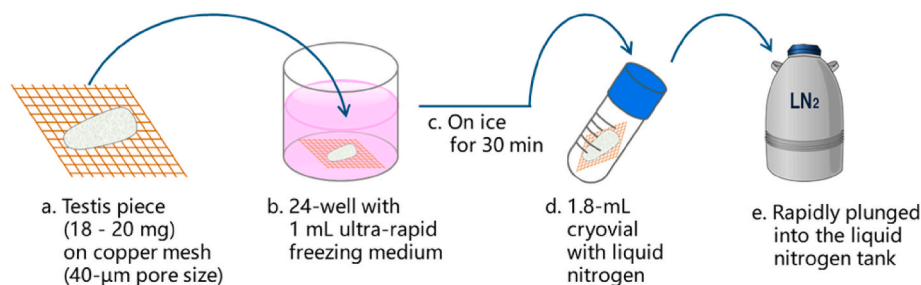


Fig. 1. Schematic representation of the ultra-rapid freezing process for testicular tissue in *M. rosenbergii*. a. A testis piece was placed on a copper mesh of 40- μ m pore size. b. The mesh with the testis piece was transferred into a 24-well plate containing 1 mL of ultra-rapid freezing medium. c. The plate was incubated on ice for 30 min. d. The mesh with the testis piece was transferred into a 1.8-mL cryovial pre-filled with liquid nitrogen. e. The cryovial was plunged into a liquid nitrogen tank.

and 15 % ME₂SO. After one week of storage in liquid nitrogen, the testicular cells were prepared, and the viability and recovery of spermatogonia that had been preserved by slow freezing and ultra-rapid freezing were examined as described above. Freshly prepared testis

pieces were used as the control. Five replicates using five specimens each were used for each set of conditions.

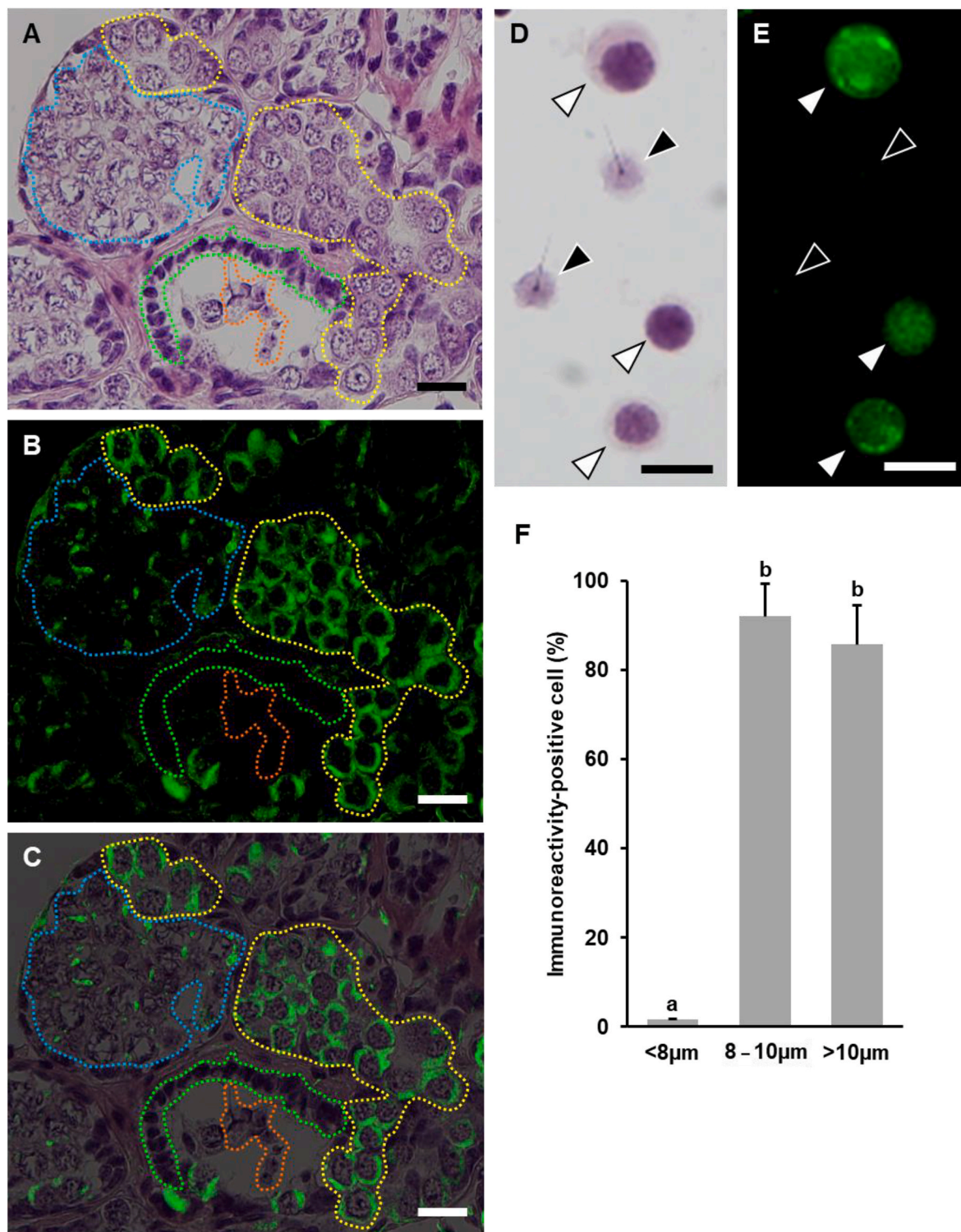


Fig. 2. Immunohistochemical analysis of testis section (A–C) and immunocytochemical analysis of dissociated testicular cells (D–F) using a commercially available anti-zebrafish vasa antibody of *M. rosenbergii*. Paraffin sections of testes were (A) stained with hematoxylin and eosin or (B) anti-zebrafish vasa antibody. (C) merged images, (D, E) dispersed testicular cells with immunohistochemical staining in (D) bright view with HE staining and (E) fluorescent view. Δ Immunoreactivity-positive spermatogonia >8 μm diameter; ▲ Immunoreactivity-negative cells <8 μm diameter. (F) frequency (%) of immunoreactivity-positive cells with diameters of <8 μm, 8–10 μm, and >10 μm. Bars represent mean ± SE (n = 4). Values with different letters are significantly different (P < 0.05). Scale bars: A – C, 20 μm; D – E, 10 μm.

2.11. Optimization of equilibration time for slow freezing method

To optimize the time for the cells to be equilibrated with cryoprotectants before slow freezing [45], we incubated the testis pieces in 10 % ME₂SO on ice for 15, 30, or 60 min before cooling them at 1 °C/min for 90 min in a Bicell freezing container held at −80 °C and then stored them in liquid nitrogen. After a week, the pieces were thawed in a water bath at 10 °C and dissociated as described above. The viability and recovery of spermatogonia were then evaluated. Freshly prepared testis pieces were used as the control. Five replicates using five specimens each were used for each set of conditions.

2.12. Effect of thawing temperature on viability

Slow-frozen testis pieces, stored in liquid nitrogen for one week with 10 % ME₂SO as cryoprotectant, were thawed in a water bath at 10 °C or 27 °C for 1 min, then transferred to the supplemented L-15 medium. Ultra-rapidly frozen testis pieces stored in liquid nitrogen for one week were thawed by direct immersion in supplemented L-15 medium at 10 °C or 27 °C. The testicular cells were then prepared, and their viability and recovery were evaluated as described above. Freshly prepared testis pieces were used as the control. Five replicates using five specimens each were used for each set of conditions.

2.13. Effect of long-term cryopreservation

Testis pieces were cryopreserved by slow freezing and ultra-rapid freezing in 10 % ME₂SO. At 1 and 6 months after cryopreservation, the slow-frozen pieces were thawed at 10 °C in a water bath, and the ultra-rapidly frozen pieces were thawed by direct immersion in supplemented L-15 medium at 10 °C. The thawed pieces were enzymatically dissociated, and the viability and recovery of spermatogonia were investigated as above. Freshly prepared testis pieces were used as the control. Five replicates using five specimens each were used at each time point.

2.14. Statistical analysis

The results are expressed as mean ± standard deviation (SD) or standard error (SE). Data were analyzed by repeated-measures one-way analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparison test to determine the significance of differences between group means. For experiments with two sample groups such as optimization of thawing temperature, two-sample *t*-test analysis was performed. Statistical significance was set at *P* < 0.05.

3. Results

3.1. Germ cell identification

The developmental stages of germ cells were classified as spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatozoa based on their morphology, as described in a previous study [33,38]. In this study, it proved difficult to distinguish the stages of spermatogonia (type A, type B, or differentiating) based on their morphology; we therefore collectively referred to them as spermatogonia. Immunohistochemical analysis with commercially available anti-zebrafish vasa antibody revealed strong immunoreactivity in spermatogonia (the yellow-bordered cells in Fig. 2A–C), weak immunoreactivity in primary spermatocytes (the blue-bordered cells in Fig. 2A–C), and no detectable immunoreactivity in secondary spermatocytes (the green-bordered cells in Fig. 2A–C) or spermatozoa (the orange-bordered cells in Fig. 2A–C).

Immunocytochemistry of dissociated testicular cells followed by cell size determination showed that about 90 % of cells with a diameter of 8–10 µm were immunoreactivity-positive spermatogonia (Fig. 2D–F).

The commercially available anti-zebrafish vasa antibody used in this study detected a prominent band at approximately 50 kDa and a faint band at around 45 kDa in Western blotting (Fig. 3), although the predicted molecular weight of the *M. rosenbergii* Vasa protein is approximately 66.46 kDa.

3.2. Optimization of cryoprotectant

The recovery rates of spermatogonia after slow freezing were highest in ME₂SO (75.6 %), followed by glycerol and MgCl₂, with ME₂SO showing significantly higher recovery than MgCl₂ (Fig. 4B). Their viability after slow freezing in the ME₂SO group (87.8 %) was not significantly different from that for fresh tissue (control, 91.9 %), while viability in glycerol and MgCl₂ was significantly lower than seen with fresh tissue (Fig. 4C).

For the ultra-rapid freezing method, the recovery rate of spermatogonia was similarly higher in ME₂SO (71.8 %) and glycerol (71.0 %) than in MgCl₂ (Fig. 4D) and their viability in ME₂SO (90.9 %) was significantly higher than in glycerol and MgCl₂ (Fig. 4E).

3.3. Optimization of ME₂SO concentration

Because viability and recovery were the highest in ME₂SO, we optimized its concentration. After slow freezing, recovery was highest in 10 % ME₂SO (75.6 %), significantly higher than in 15 % ME₂SO but not higher than in 5 % ME₂SO (Fig. 5A). Although viability was highest in 5 % ME₂SO (97.0 %) compared to 10 % and 15 % ME₂SO (Fig. 5B), the mean recovery rate in the 10 % ME₂SO group was markedly higher than that in the 5 % ME₂SO group. Based on these results, the optimal concentration of ME₂SO for the slow freezing method was determined to be 10 %.

For the ultra-rapid freezing method, recovery was significantly higher in 10 % ME₂SO (83.4 %) than in 5 % and 15 % ME₂SO (Fig. 5C). Viability in the 10 % ME₂SO group (88.6 %) was as high as that in the 5 % ME₂SO group and significantly higher than in the 15 % ME₂SO group (Fig. 5D). These findings showed, similar to the slow freezing method, 10 % to be the most effective concentration of ME₂SO for the ultra-rapid freezing method.

3.4. Optimization of equilibration time for the slow freezing method

Both recovery and viability declined slightly as the equilibration

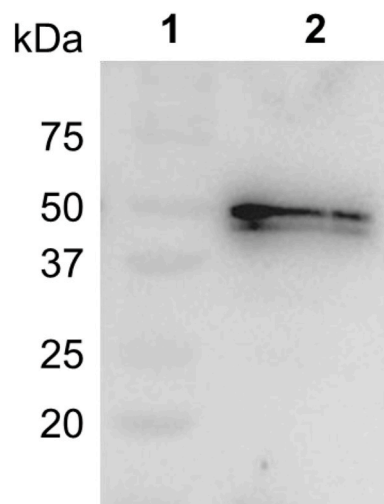


Fig. 3. Western blot analysis of testis extracts using anti-zebrafish vasa antibody. A commercially available anti-zebrafish vasa antibody showed 2 bands with a molecular mass of approximately 50 and 45 kDa in the extracts of testis of *M. rosenbergii*. Lane 1, size marker. Lane 2, testis extracts.

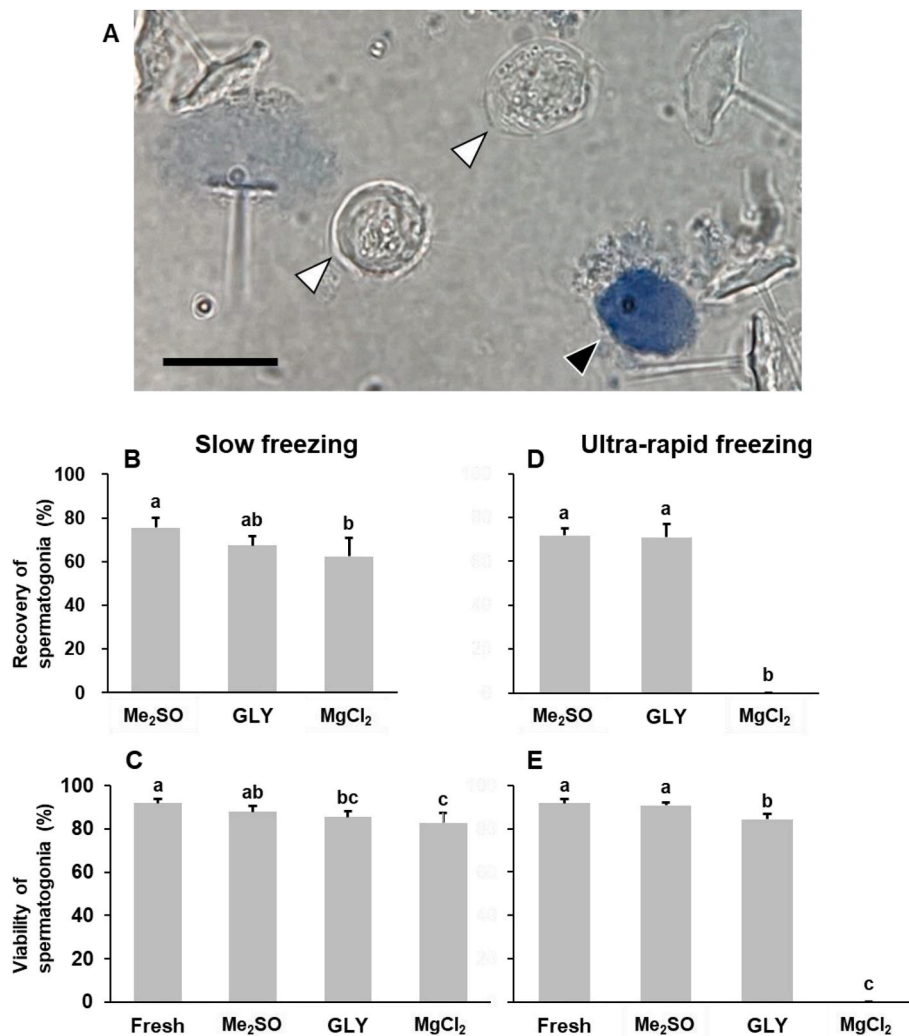


Fig. 4. Evaluation of different cryoprotectants during slow freezing (B, C) and ultra-rapid freezing (D, E) of the testis of *M. rosenbergii*. (A) Dispersed testicular cells stained with trypan blue. Δ Unstained live cells; \blacktriangle stained dead cell. Scale bar: 10 μ m. (B–E) Recovery and viability of frozen–thawed spermatozoa in 10 % ME₂SO, 10 % glycerol (GLY), or 10 % MgCl₂ in the (B, C) slow freezing and (D, E) ultra-rapid freezing methods. Bars represent mean \pm SD ($n = 5$). Values with different letters are significantly different ($P < 0.05$).

time increased (Fig. 6). Both were highest, though not significantly, at 15 min (96.5 % and 97.5 % in recovery and viability, respectively), followed by 30 min and 60 min. Viability declined marginally from the freshly prepared control (98.2 %) to 60 min (94.3 %).

3.5. Optimization of thawing temperature

After slow freezing, both recovery and viability exceeded 90 % when thawed at 10 °C and were significantly higher than at 27 °C (Fig. 7A and B). With the ultra-rapid freezing method, recovery was significantly higher at 10 °C (89.6 %) than at 27 °C (57.9 %) (Fig. 7C), while viability showed no significant difference between temperatures, being slightly higher at 10 °C (91.4 %) than at 27 °C (88.3 %) (Fig. 7D). These findings showed a thawing temperature of 10 °C to be more suitable than 27 °C for both slow freezing and ultra-rapid freezing.

3.6. Long-term cryopreservation

Recovery and viability were significantly higher when using ultra-rapid freezing than after slow freezing following 1, 2, 3, and 6 months of cryopreservation (Fig. 8A and B). Notably, with ultra-rapid freezing, recovery remained at above 86 %, and survival exceeded 92 % even after six months of cryopreservation. In contrast, slow freezing showed lower

performance, with recovery at approximately 51 % and survival at around 84 % after six months. With the ultra-rapid freezing method, both recovery and survival declined slightly but not significantly over time (Fig. 8A and B). After slow freezing, in contrast, recovery had declined significantly at 6 months and viability at 2 and 3 months (Fig. 8A and B).

4. Discussion

This study successfully established protocols for cryopreserving spermatozoa of the freshwater prawn *Macrobrachium rosenbergii* using both slow freezing and ultra-rapid freezing. The high recovery and viability rates of the cryopreserved spermatozoa after thawing confirmed the type and concentration of cryoprotectants, equilibration time, and thawing temperature to have been effectively optimized. In a previous study, we developed cryopreservation protocols for the spermatozoa of banana shrimp (*Fenneropenaeus merguensis*) and black tiger shrimp (*Penaeus monodon*) [40]. This study brings to three the number of economically important shrimp species for which cryopreservation protocols for spermatozoa have been established, paving the way for creating a cryopreservation bank for established strains of shrimp that have valuable traits. However, freshwater prawns, even within the same species, are known to exhibit different

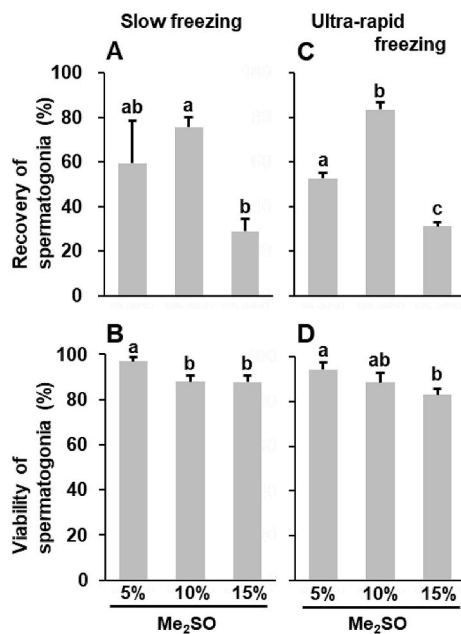


Fig. 5. Effects of ME₂SO concentration on recovery (A, C) and viability (B, D) of spermatozoa during slow freezing (A, B) and ultra-rapid freezing (C, D) in *M. rosenbergii*. (A, C) Recovery and (B, D) viability of frozen-thawed spermatozoa in 5 %, 10 %, or 15 % ME₂SO as cryoprotectant used in the (A, B) slow freezing and (C, D) ultra-rapid freezing methods. Bars represent mean \pm SD ($n = 5$). Values with different letters are significantly different ($P < 0.05$).

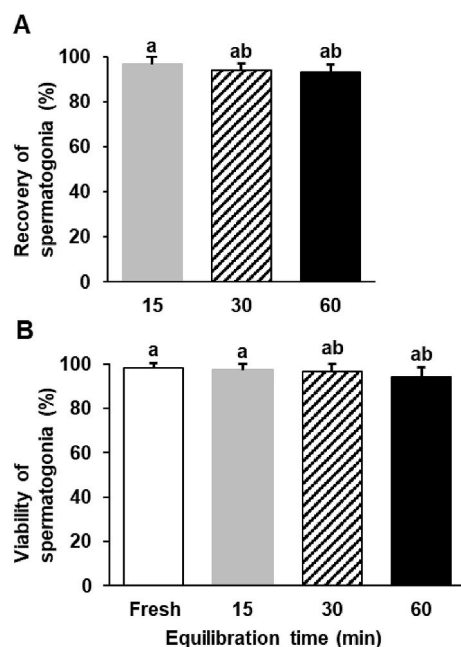


Fig. 6. Effects in *M. rosenbergii* of equilibration time on the recovery (A) and viability (B) of spermatozoa before slow freezing. (A) Recovery and (B) viability of frozen-thawed spermatozoa equilibrated before immersion in liquid nitrogen for 15, 30 and 60 min on ice with 10 % ME₂SO. “Fresh” in B indicates freshly prepared testis pieces used as the control. Bars represent mean \pm SD ($n = 5$). Values with different letters are significantly different ($P < 0.05$).

physiological and/or reproductive characteristics across regional populations [19,20,46,48], which may have influenced the results observed in this study. It should therefore be noted that the findings reported here may primarily reflect the characteristics of the regional population of *M. rosenbergii* in Songkla Lake, as the individuals used in

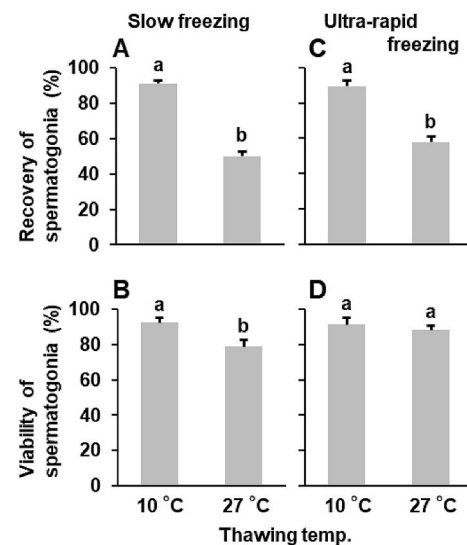


Fig. 7. Effects in *M. rosenbergii* of thawing temperature on recovery (A, C) and viability (B, D) of frozen spermatozoa in slow freezing (A, B) and ultra-rapid freezing (C, D) methods. (A, C) Recovery and (B, D) viability of frozen-thawed spermatozoa when thawed at 10 °C or 27 °C in (A, B) slow freezing and (C, D) ultra-rapid freezing methods. Bars represent mean \pm SD ($n = 5$). Values with different letters are significantly different ($P < 0.05$).

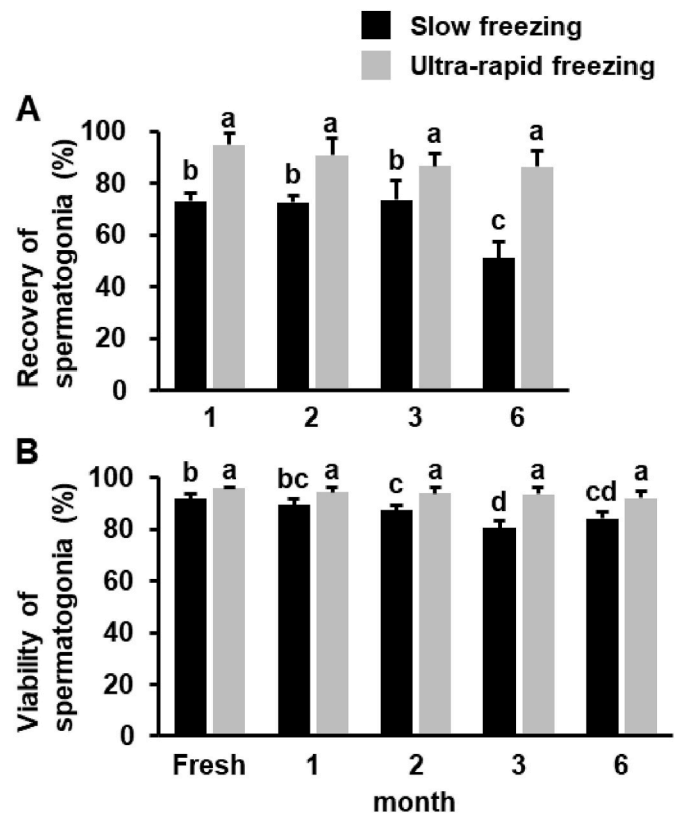


Fig. 8. Recovery (A) and viability (B) of frozen-thawed spermatozoa after long-term cryopreservation for up to six months in *M. rosenbergii*. (A) Recovery and (B) viability of frozen-thawed spermatozoa cryopreserved for 1, 2, 3, or 6 months using the slow freezing (black) or ultra-rapid freezing (gray) methods. “Fresh” in B indicates freshly prepared spermatozoa used as a control for viability. Bars represent mean \pm SD ($n = 5$). Values with different letters are significantly different ($P < 0.05$).

this study were collected from this specific area.

Our immunohistochemistry showed a commercially available anti-zebrafish vasa antibody to exhibit strong immunoreactivity to spermatogonia, and weak immunoreactivity in primary spermatocytes, but was undetectable in secondary spermatocytes and spermatozoa in *M. rosenbergii*. Our immunocytochemical analysis also revealed that approx. 90 % of the dissociated testicular cells with a diameter of 8–10 μm and 80 % of cells larger than 10 μm showed immunoreactivity. According to Poljaroen et al. [38], spermatogonia have a diameter of 8–10 μm , while spermatocytes, including both primary and secondary stages, have larger diameters ranging from 10 to 14 μm , depending on their developmental stage. By integrating our findings with this existing knowledge, spermatogonia could be identified efficiently with high probability even with only one indicator: a cell diameter of 8–10 μm . It is highly likely that the immunoreactivity-positive cells larger than 10 μm include spermatocytes and spermatogonia that are in the process of differentiating into spermatocytes or undergoing mitosis (e.g., it is widely recognized that cells grow and increase in volume during the G1 and G2 phases). The immunoreactivity-positive cells with a relatively small diameter of <8 μm may include daughter cells of spermatogonia. These hypotheses will require further verification using meiotic or cell-cycle-specific markers [34,36].

Western blotting with a commercially available anti-zebrafish vasa antibody revealed one prominent band at approximately 50 kDa and a faint band at approximately 45 kDa (Fig. 3). Given that the molecular weight of Vasa from *M. rosenbergii* is estimated to be 66.46 kDa based on its amino acid sequence (GenBank: OQ565444.1), it is likely that the antibody recognizes other proteins, such as Vasa isoforms, expressed in the male germ cells of *M. rosenbergii* rather than the Vasa protein itself. At this stage, the identity of the protein(s) recognized in this study remains unknown; however, our immunohistochemical analysis clearly demonstrated the antibody targets molecule(s) to be highly expressed in the spermatogonia of *M. rosenbergii*.

Cryopreservation protocols have been established for various crustacean species, including *M. rosenbergii* [1,5,6,50], the mud spiny lobster (*Panulirus polyphagus*) [11], Indian white shrimp (*Fenneropenaeus indicus*) [29], black tiger shrimp (*P. monodon*) [51], Chinese mitten crab (*Eriocheir sinensis*) [22], and banana shrimp (*F. merguensis*) [31]. Although these studies focus on the preservation of spermatophores and spermatozoa rather than spermatogonia, their use of ME_2SO , glycerol, ethylene glycol, propylene glycol, and methanol as cryoprotectants parallels our recent research on cryopreservation techniques for spermatogonia in banana shrimp (*F. merguensis*) and black tiger shrimp (*P. monodon*) in which we demonstrated the effectiveness of ME_2SO and glycerol as cryoprotectants [40]. Collectively, these findings suggest that ME_2SO and glycerol are suitable cryoprotectants for germline cryopreservation in a wide range of shrimp species, regardless of whether they be freshwater or saltwater species, for spermatophores, spermatozoa, and spermatogonia. When developing germ cell cryopreservation techniques for other shrimp species, it may be beneficial to initially optimize conditions based on the use of ME_2SO and glycerol.

ME_2SO has been reported as an effective cryoprotectant in aquatic invertebrates [2,12], as here. However, it is also known to exhibit cytotoxic effects on certain cell types [8,9]. The use of 15 % ME_2SO may have exerted toxic effects on the spermatogonia of *M. rosenbergii*, potentially accounting for the reduced recovery and viability observed at this concentration compared to 5 % and 10 % seen in this study. Although copper is known to be potentially toxic to some marine invertebrates [16], no adverse effects were observed in our study using copper grids during the short exposure period; however, further studies are needed to evaluate the ability of the thawed germ cells to proliferate and differentiate, particularly in future applications of transplantation, which remains under development.

Events such as recrystallization, cell hydration, and intracellular devitrification occur during the thawing of frozen cells, potentially

causing significant damage if not managed [23]. Here, the recovery (in particular) and viability rates of frozen-thawed spermatogonia were higher when thawed at 10 °C than at 27 °C. Similar results were observed in our study on marine shrimp [40]. Meanwhile, research on *Macrobrachium* species, including *M. rosenbergii* and *M. acanthurus*, which has primarily focused on the cryopreservation of spermatophores rather than germ cells, has shown that thawing preserved spermatophores at 30 °C or 35 °C results in spermatozoa with good viability and activity [1,5–7,50]; as reviewed by Aquino et al. [2]. The differences in size and morphology between spermatogonia and spermatozoa may explain why thawing temperature affects them differently.

Based on previous findings [41,42], the mean GSI of 0.27 % observed in this study corresponds to a reproductive stage at which spermatocyte production in the testes is actively increasing. At the same time, a wide variation in the gonad-somatic index (GSI, 0.09 %–0.54 %) was observed among the individuals used in this study, indicating the diverse reproductive statuses of their testes. In fact, histological analyses have revealed that some testes, typical of immature stages, were predominantly occupied by spermatogonia, while others, typical of mature stages, were largely occupied by spermatozoa, and still others exhibited intermediate reproductive states. Previous studies on germ cell transplantation in fish have furthermore reported that spermatogonia, even when prepared from testes at various reproductive stages, can be successfully used for transplantation experiments, regardless of the maturity stage of the testes [24,43,44,53]. Due to this evidence, the reproductive status of the testes was not specifically considered in this study. On the other hand, spermatogonia can be classified into type A, type B, and differentiating stages. Notably, in germ cell transplantation in fish, differences in the success rates of incorporation into the recipient's gonad have been observed not only between type A and type B spermatogonia but even among type A spermatogonia [13,52]. These findings highlight the fact that spermatogonia can be classified into distinct sub-populations according to their function. However, in shrimp, there are as yet no methods for distinguishing and isolating spermatogonial stages in live cells other than fixation-based morphological identification. Future studies on shrimp will likely need to focus on identifying molecular markers that distinguish spermatogonial stages, which could then be utilized to conduct functional assays and comparisons of spermatogonia across different stages, particularly for use in transplantation.

Building on these findings, it is noteworthy that germ cell transplantation technology has been established for many fish species, and by combining this technology with germ cell cryopreservation technology, it has been possible to preserve the genetic information of endangered species and valuable strains [17,18,25–27,32,39,44,55–57]. If an endangered species becomes extinct or valuable strains are lost, for example, frozen germ cells can be thawed and transplanted into a recipient to restore such species or strains. Most recently, germ-cell transplantation techniques have been developed for shrimp [4]. Together with the cryopreservation techniques established here, this technology holds immense potential to minimize the risk of losing valuable strains and allows for more efficient seed production in shrimp within a shorter time frame and in a smaller space.

CRedit authorship contribution statement

Tomoyuki Okutsu: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Conceptualization. **Natthida Rakbanjong:** Visualization, Validation, Investigation, Formal analysis. **Shinya Shikina:** Writing – review & editing, Writing – original draft. **Misako Miwa:** Visualization, Validation, Investigation. **Monwadee Wonglaphsuwan:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 4o in order to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of interests

None.

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