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SPERM CRYOPRESERVATION OF GIANT GROUPER *Epinephelus lanceolatus* USING TUALANG HONEY AS A NATURAL ALTERNATIVE CRYOPROTECTIVE AGENT

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ABSTRACT

The current sperm cryopreservation protocol for *Epinephelus lanceolatus* uses Dimethyl Sulfoxide (DMSO) as a Cryoprotective Agent (CPA), which may exhibit toxicity at high concentrations. To address this limitation, this study investigated Tualang Honey (TH) as a natural, non-toxic alternative CPA. Specifically, this study aimed to determine the best concentration of TH and TH mixed with DMSO (THD), as well as the optimum cooling protocol when using TH as a CPA in the sperm cryopreservation protocol of *E. lanceolatus*. We used five concentrations (5%, 10%, 15%, 20%, and 25%) of TH and five combinations (5%, 10%, 15%, 20%, and 25%) of THD (1:1 TH:DMSO) as CPA. The cooling protocol using TH was optimised by manipulating the cooling heights (3, 5, 7, and 9 cm) during the cooling process. Sperm Post-Thaw Motility (PTM) and motility duration were used to evaluate sperm quality. All data were presented as mean ± Standard Error (SE) and analysed using one-way Analysis of Variance (ANOVA, $p < 0.05$) followed by Tukey's post hoc test. The results revealed that all concentrations of TH and THD were not significantly different ($p > 0.05$) from the control (42.00% ± 10.0%). Among all treatments, 20% TH and 15% THD yielded the highest PTM values (29.33% ± 1.33% and 25.67% ± 5.17%, respectively). Furthermore, the highest PTM value was obtained when cooling at 7 cm above the Liquid Nitrogen (LN) surface (42.00% ± 1.00%). Overall, the results suggest that TH and THD can be used as alternatives to CPA for sperm cryopreservation of *E. lanceolatus, with* 20% TH providing the highest PTM values.

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Introduction

The hybrid grouper, *Epinephelus fuscoguttatus* × *Epinephelus lanceolatus,* is an important aquaculture species due to the high demand, especially in the Far East and Southeast Asia (FAO, 2000; Rimmer & Glamuzina, 2019; Nankervis *et al.*, 2022). Consequently, the giant grouper, *E. lanceolatus*, is a particularly important broodstock, as male sperm is required to produce the hybrid grouper. However, *E. lanceolatus* is a protogynous hermaphrodite that first matures as a female before changing sex to male at very large sizes. As a result, the availability of synchronously mature males is limited, making sufficient spermatozoa production difficult (Rurangwa *et al.*, 2004). Moreover, wild broodstock of *E. lanceolatus* are difficult to find and obtain, as male groupers are typically larger, older, and less common than females (Heemstra & Randall, 1993). In contrast, domesticated fish sources require a long rearing and culture period before they are suitable as broodstock. These challenges are further compounded by the dramatic decline of wild giant grouper range and numbers due to human activity such as overfishing and habitat degradation. In this regard, the species are currently designated as data-deficient (Fennessy *et al.*, 2018). Additionally, the large size of males makes them difficult and costly to maintain and manage in captivity.

Sperm cryopreservation therefore supports gamete collection from *E. lanceolatus* for aquaculture production of hybrid grouper (Yusoff *et al.*, 2018). Sperm cryopreservation preserves male fish gametes at cryogenic temperatures (-196°C) for long-term storage (Muchlisin *et al.*, 2005). Sperm cryopreservation provides various advantages, including long-term preservation (Ashwood-Smith, 1980; Fabbrocini *et al.*, 2015; Idris *et al.*, 2025), genetic management of broodstock, and improved artificial insemination efficiency in aquaculture (Magnotti *et al.*, 2018; Che-Zulkifli *et al.*, 2020). In spermatozoa cryopreservation, the function of Cryoprotective Agent (CPA) is to protect spermatozoa against damage caused by freezing. CPAs prevent intracellular ice crystal formation and excessive dehydration (Pegg, 2007). Building on this, Dimethyl Sulfoxide (DMSO) is the most commonly used CPA in sperm cryopreservation. However, it is highly toxic to sperm at high concentrations, especially if thawing and fertilisation protocols are not clearly optimised, resulting in reduced sperm motility and sperm cell mortality, which can cause Deoxyribonucleic Acid (DNA)–methylation and histone alteration (Yong *et al.*, 2015).

Due to CPA toxicity, non-toxic CPAs are needed as an alternative to store cells at Liquid Nitrogen (LN) temperature. In this study, Tualang Honey (TH) is used as an alternative or substitute sperm CPA for the cryopreservation of *E. lanceolatus*. TH is a multiflora honey that is obtainable from the tropical jungles of Peninsular Malaysia, Southern Thailand, Northern Sumatra, and Borneo, and is produced by the species *Apis dorsata,* which is known as the Giant Asian rock bee. Although the cryoprotective properties of honey have been demonstrated in human cells and tissues, empirical support for its efficacy in preserving fish sperm remains limited (Cheepa *et al.*, 2022). Honey also contains high sugar concentrations, which may safeguard sperm cells from the

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detrimental effects of cryopreservation (Alfoteisy *et al.*, 2020).

This study aims to investigate the potential of TH as a replacement for DMSO as a CPA or as a supplement to increase the cryopreservation capability. This study also seeks to identify the best cooling protocol when using TH and help provide fish breeders with an easy-to-use cryopreservation protocol that uses natural CPAs that are easier to obtain. Specifically, this study aims to identify the optimal TH concentration, the best TH concentration when mixed with DMSO as a CPA, and to optimise the cooling protocol for *E. lanceolatus* spermatozoa cryopreservation using TH.

Materials and Method

Broodstock Management and Sperm Collection

Sperm of *E. lanceolatus* was collected from mature males (n = 3) selected from the net cages at Pulau Ketam. Sperm was collected by stripping and stored in microcentrifuge tubes. The sperm was mixed with extender at a 1:1 ratio (sperm to extender) and immediately stored in a cooler box containing crushed ice (1°C to 4°C). Next, the sperm sample was transferred to Universiti Malaysia Terengganu (UMT) by courier.

Initial Sperm Quality Evaluation

Sperm quality was evaluated before any experiments or analysis. For the initial sperm motility evaluation, 1 µL was diluted with 999 µL of seawater to activate the sperm, then immediately transferred to a glass slide and examined under a microscope (Olympus CX10; negative phase contrast objective lens, 20 ×). Following this, motility was recorded using a video camera (Dino eye) with a minimum acquisition time of 2 s, modified from Koh *et al.* (2010), until all sperm were immotile. Subsequently, motility duration was determined by observing sperm movement until all sperm were immotile. The motility (%) of sperm was evaluated using the following formula (Idris *et al.*, 2024):

$$\text{Motility (\%)} = [\text{Motile sperm} \div (\text{Total sperm cells observed})] \times 100$$

Experiment 1: Optimum Concentration of Tualang Honey (TH) as a Cryoprotectant

In Experiment 1, the effect of five different concentrations (5%, 10%, 15%, 20%, and 25%) of TH as a CPA on sperm motility (%) and duration of sperm cryopreservation of *E. lanceolatus* was evaluated. DMSO at 10% concentration was used as a control in this experiment. The various TH concentrations were mixed with Modified Plaice Ringer Solution (MPRS), consisting of 60.35 mM NaCl, 1.8 mM NaH_2PO_4, 5.23 mM KCl, 3.0 mM $NaHCO_3$, 1.13 mM $CaCl_2.2H_2O$, 1.13 mM $MgCl_2.6H_2O$, and 55.55 mM D-glucose. In addition, the sperm were mixed with cryodiluent at a 1:9 sperm-to-cryodiluent ratio (Horvath *et al.*, 2003). After mixing the sperm with cryodiluent, the sperm sample was pipetted into 0.25 mL cryostraws and sealed with straw powder. The cryostraws were then equilibrated at room temperature for 3 min before freezing with methods modified from Yang *et al.* (2022).

Freezing was conducted using an experimental dewar containing LN at a depth of 8 cm, with methods modified from Idris *et al.* (2024). The cryostraws were placed on hollow styrofoam blocks at a height of 7 cm above the surface of LN for one and a half

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minutes, then immediately immersed in LN. Following this, the cryostraws were transferred to the LN tank storage for 24 h before evaluating sperm's Post-Thaw Motility (PTM) and duration.

Experiment 2: Optimum Concentration of Tualang Honey Mixed with DMSO (THD) as a Cryoprotectant

In this experiment, the method was similar to Experiment 1, except for the type of cryodiluent used. Five concentrations of 1: 1 TH + DMSO cryodiluents (THD) (5%, 10%, 15%, 20%, and 25%) were evaluated, with 10% DMSO used as a control. The concentration of CPA consisted of TH and DMSO at a ratio of 1: 1. Therefore, the concentration of 5% refers to 2.5% of DMSO and 2.5% of TH, and so forth.

Experiment 3: Optimum Cooling Rate of Tualang Honey (TH) as a Cryoprotectant

TH at 20% concentration was used as a CPA. The cooling protocol was similar to that of Experiments 1 and 2. However, for this experiment, the cryostraws were placed at four different heights (3, 5, 7, and 9 cm) to cool from the LN surface and were cooled for 1 min 30 s in the Dewar. The different heights manipulated the cooling rate. The closer the cryostraw was to the LN surface, the faster the cooling rate. The cryostraw was then fully immersed in LN and transferred to the LN tank for at least 24 h before thawing and observing sperm PTM and duration.

Post-Thaw Sperm Quality Evaluation

Cryostraws that had already been stored in the LN tank for 24 h were removed from the tank. The cryostraws were then thawed in a room-temperature water bath for 1 min 30 s. Subsequently, cryopreserved sperm were transferred from the straw to a microcentrifuge tube and mixed. Next, 10 µL of cryopreserved sperm was pipetted and mixed with 990 µL of seawater to activate sperm motility (final dilution ratio of 1,000). In addition, 10 µL of activated sperm was pipetted onto the glass slide and video-recorded under the electronic microscope using Dino-Eye camera software. Video recording began immediately when sperm started mixing with seawater. Sperm motility and duration were concurrently analysed from the video as described previously, following methods modified by Idris *et al.* (2024).

Data Analysis

All data were assessed for normality before analysis. Differences in sperm motility percentage and motility duration were analysed using one-way Analysis of Variance (ANOVA). Where significance was detected, Tukey's post hoc test was subsequently performed to determine differences among treatments at a significance level of $p < 0.05$. All data were presented as mean ± Standard Error (SE). Statistical analyses were performed using SPSS 27.0 software (SPSS, Chicago, IL, United States of America).

Results and Discussion

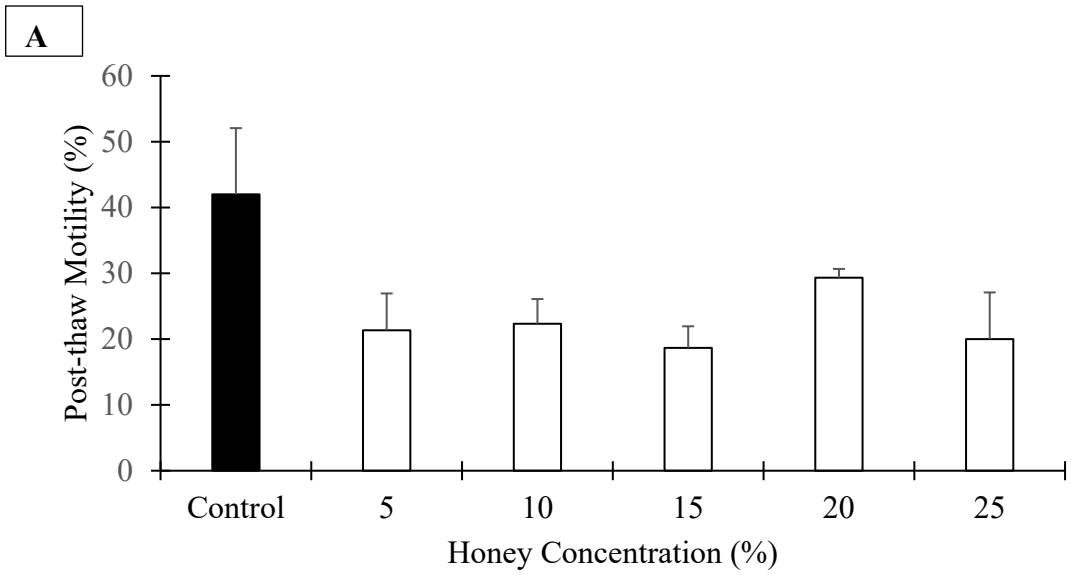
Tualang Honey as Cryoprotective Agent

E. lanceolatus weighs up to 400 kg and is a protogynous hermaphrodite, which leads to a lack of synchronously mature males. Sperm from this species is difficult to obtain and is highly important for hybridisation with other grouper species. Sperm cryopreservation of *E. lanceolatus* is important given this species spawns seasonally, with spawning only occurring once per month. The experiments used TH as a CPA in sperm cryopreservation of *E. lanceolatus*. For *E. lanceolatus*, the different concentrations of TH used as a CPA

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did not significantly affect sperm PTM. TH contains mainly sugars, with a high content of fructose (29.6%), glucose (30.0%), sucrose (0.6%), and maltose (7.6%) (Ahmed & Othman, 2013). Sugars are widely employed in cryopreservation procedures for sperm in a variety of fish species, including African catfish, *Clarias gariepinus* (Urbanyi *et al.*, 1999), common carp, *Cyprinus carpio* (Horváth *et al.*, 2003), Arctic Char, *Salvelinus alpinus* (Mansour *et al.*, 2006), kelp grouper, *Epinephelus moara* (Miyaki *et al.*, 2005), dusky grouper, *Epinephelus marginatus* (Riesco *et al.*, 2017), giant grouper, *E. lanceolatus* (Yang *et al.*, 2022), and orange spotted grouper, *Epinephelus coioides* (Peatpisut & Bart, 2010) as CPA, supplements or extenders to enhance sperm motility. However, in studies on salmonids (family Salmonidae), including brown trout, *Salmo trutta*, brook trout, *Salvelinus fontinalis*, sex-reversed rainbow trout, *Oncorhynchus mykiss* (Nynca et al., 2016), Persian sturgeon, *Acipenser persicus*, and Beluga sturgeon, *Huso huso* (Golshahi et al., 2018), sugar was not effective as a CPA. This suggests that the cryoprotective effect of various types of sugars is variable and highly species-specific.

Yang *et al.* (2022) reported that different types of sugar extender had no significant effect on the subsequent cryoprotective efficiency of brown-marbled grouper, *E. fuscoguttatus* and are thus interchangeable with each other. This is reflected in this study's results, where different concentrations of TH successfully cryopreserved *E. lanceolatus* sperm (Figure 1). In particular, PTM values were highest in the control (10% DMSO) with 42.00% ± 10.06%, followed by 20%, 10%, and 5% TH with motility values of 29.33% ± 1.33%, 22.33% ± 3.75% and 21.33% ± 5.60%, respectively. In comparison, the lowest values were observed in 15% TH with motility of 18.67% ± 3.28%. However, no significant differences ($p > 0.05$) were observed between all conditions, including control for both PTM and PTD. This implies that all TH concentrations in Experiment 1 can be used for sperm cryopreservation of *E. lanceolatus* and could directly substitute for DMSO. Likewise, the different sugar compositions in TH did not appear to affect its cryoprotective capabilities.



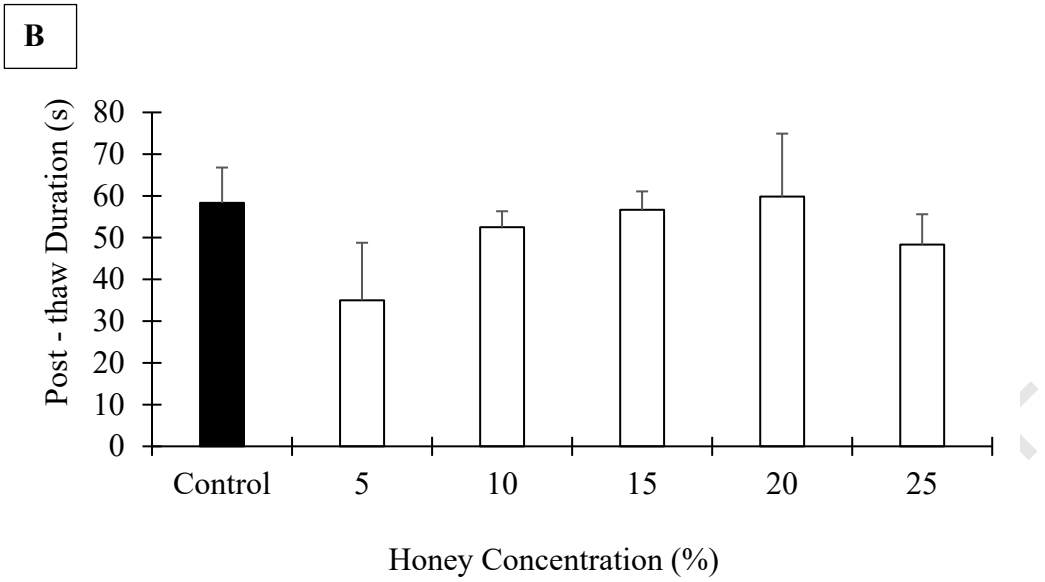


Figure 1: Sperm (A) PTM (%) and (B) post-thaw duration (s) of *E. lanceolatus* when cryopreserved with different TH concentrations (5% to 25%). No significant differences were observed between treatments ($p > 0.05$)

Combination of Tualang Honey and DMSO as Cryoprotective Agent

In the second experiment, the TH concentration was reduced and used in combination with DMSO. The results of Experiment 2 (Figure 2) present the effect of different concentrations of THD on PTM of *E. lanceolatus*. Of the six different concentrations used, including control (10% DMSO), a significant difference ($p < 0.05$) between PTM of control and 25% THD was observed. Nonetheless, no significant differences ($p > 0.05$) were observed between 5%, 10%, 15%, and 20% THD and the control. The highest PTM percentage was in the control with 42.00% ± 10.07%, while the lowest result in this experiment was detected in 25% THD with PTM of 11.00% ± 4.33%. Meanwhile, PTD values were highest in 15% THD (59.16 ± 7.95 s), followed by control (58.33 ± 8.46 s), 5 (51.67 ± 5.46 s), 10 (51.67 ± 12.02 s), 20 (49.17 ± 7.94 s), and 25% THD (44.17 ± 5.46 s). Still, no differences ($p > 0.05$) were observed for motility duration between concentrations used in this experiment, and PTD ranged from 44.17 ± 5.46 s to 59.16±7.95 s.

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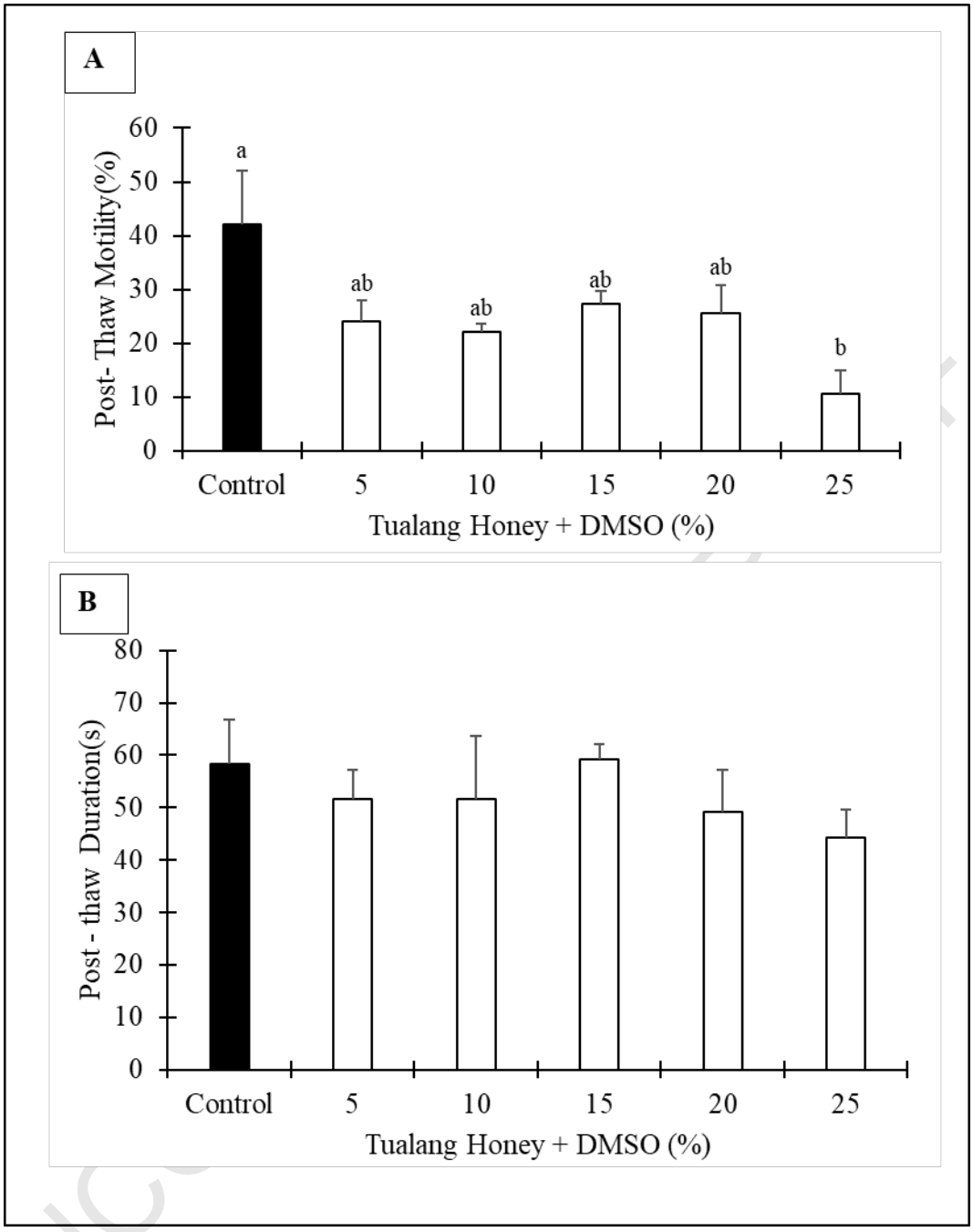


Figure 2: Sperm (A) PTM (%) and (B) post-thaw duration (s) of *E. lanceolatus* when cryopreserved with different THD concentrations (5% to 25%). Significant differences between treatments are noted in different subsets ($p > 0.05$)

DMSO is the most used CPA for cryopreservation of marine sperm species (Cabrita *et al.*, 2009; Magnotti *et al.*, 2018; Che Zulkifli *et al.*, 2020; Hema & Koh, 2021). It has been applied widely to the genus *Epinephelus* as an effective CPA, such as in giant grouper, *E. lanceolatus* (Yang *et al.*, 2022), greasy grouper, *Epinephelus tauvina* (Withler & Lim, 1982), black grouper, *Epinephelus malabaricus* (Gwo, 1993), kelp grouper, *Epinephelus bruneus* (Imaizumi *et al.*, 2005), dusky grouper, *E. marginatus* (Cabrita *et al.*, 2009; Riseco *et al.*, 2017), and seven-band grouper, *Epinephelus septemfasciatus* (Koh *et al.*, 2010). However, DMSO damages sperm cells by changing

osmotic pressure, and cryopreservation severely reduces cell viability (Cabrita *et al.*, 2009; Riesco *et al.*, 2017). Similarly, using a low concentration of CPA in sperm cryopreservation may also cause ice crystal formation in the sperm cells and potentially lead to damage during freezing and thawing (Koh *et al.*, 2010). DMSO functions as a permeating CPA. In this sense, adding honey, which contains many sugars and antioxidants, in this study should complement the cryoprotective effects of DMSO. While previous studies have used sugars in combination with DMSO in the cryodiluent to supplement and improve cell resistance to freezing and thawing, the results have not been conclusive (Horváth *et al.*, 2003; Cabrita *et al.*, 2011; Riesco *et al.*, 2017).

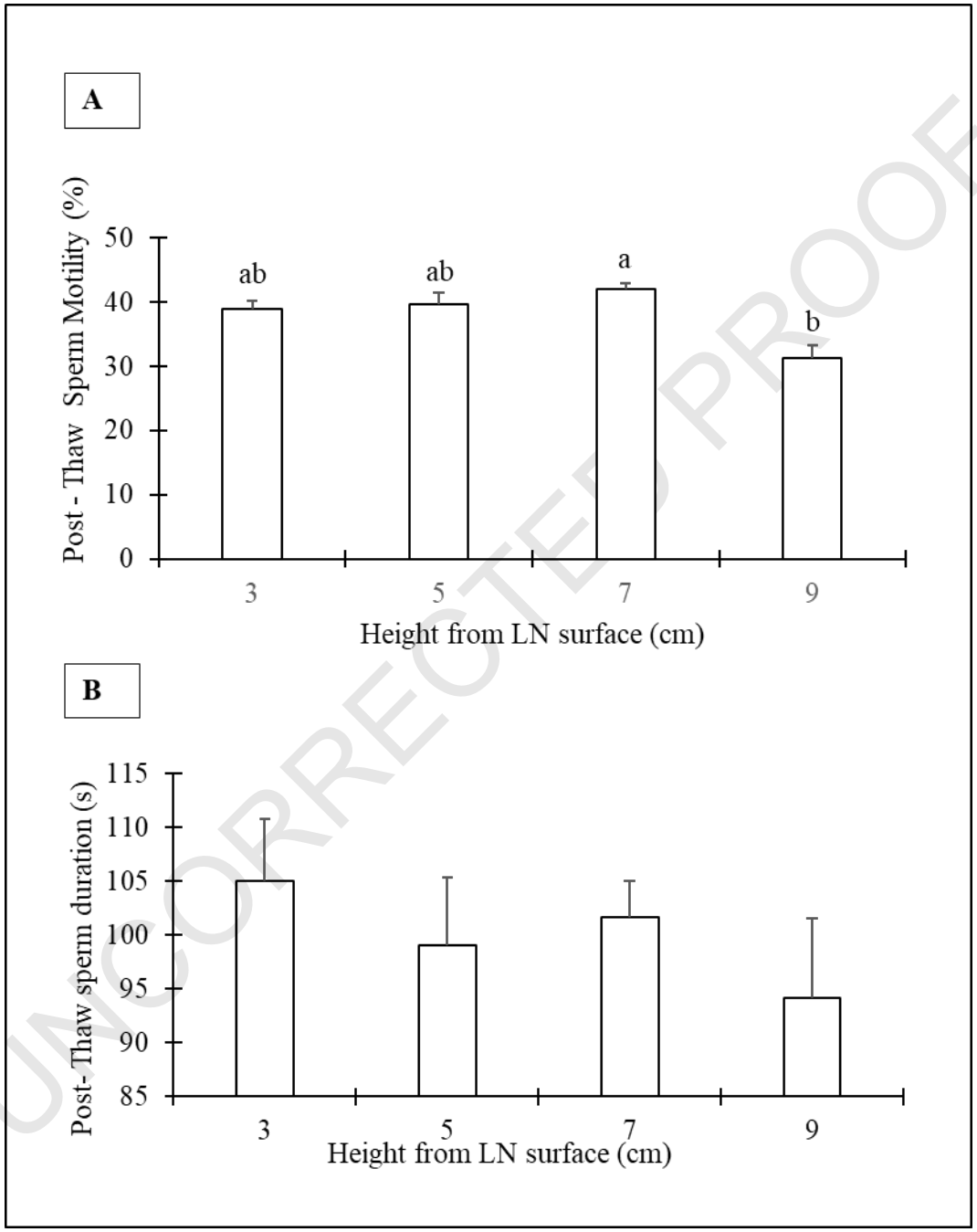
Different CPA concentrations used in sperm cryopreservation can affect the duration of sperm motility after post-thawing. High CPA concentrations in sperm cryopreservation can prevent ice crystal formation and cellular damage during cryopreservation (Koh *et al.*, 2010; Idris *et al.*, 2024). However, CPA, when used at overly high concentrations, might be toxic to the sperm and cause low motility and viability. This study found that the different concentrations used in both experiments did not significantly affect sperm PTM duration. Notably, sperm motility duration is affected by Adenosine Triphosphate (ATP) content, which can be detected based on membrane damage. Thus, damage to sperm structure may damage ATP or the tail, which can affect motility duration (Yong *et al.*, 2015). Reactive Oxygen Species (ROS) are a cause of cryodamage (Wang et al., 1997; Ball, 2008; Pérez-Cerezales et al., 2009), and the antioxidant content of TH may help protect cells from oxidative damage. TH also contains compounds such as polyphenolics, organic acids, vitamins, catalase, and glutathione peroxidase that also act as antioxidants (Aljadi *et al.*, 2004; Baltrušaitytė *et al.*, 2007; Mohamed, 2010; Shamsudin, 2022). Furthermore, honey has been reported to considerably inhibit oxidation of cell membranes and prevent cellular damage (Erejuwa *et al.*, 2012; Riesco *et al.*, 2017). TH contains high antioxidant levels, allowing it to replace DMSO as a CPA in this species. It also contains high levels of phenolic, flavonoid, and antioxidant compounds, as well as ascorbic acid, which has been established to positively affect sperm cryopreservation outcomes (Ahmed *et al.*, 2013). The concentration range used in this experiment is likely below the toxicity threshold for *E. lanceolatus* sperm. In essence, this suggests that *E. lanceolatus* sperm was more resistant to honey toxicity in terms of membrane damage, resulting in post-thaw sperm ATP content similar to that of pre-freezing sperm.

Optimal Cooling Protocol for TH-based Cryoprotective Agent

To optimise the cooling rate using TH, sperm were cryopreserved at different heights (3 to 9 cm) with a fixed cooling time of 1 min 30 s to vary the cooling rate. Lower heights resulted in faster cooling rates during sperm cryopreservation (Yusoff *et al.*, 2018; Idris *et al.*, 2024). Cooling rates at heights of 3, 5, 7, and 9 cm were 76.92°C/min, 61.54°C/min, 30.77°C/min, and 15.38°C/min, respectively (Idris *et al.*, 2024). Meanwhile, PTM presented no significant differences among 3, 5, and 7 cm heights (Figure 3), though it was highest at 7 cm, followed by 5 cm, 3 cm, and finally 9 cm, with values of 42.00% ± 1.00%, 39.67% ± 1.85%, 39.00% ± 1.15%, and 31.3% ± 2.02%, respectively. Sperm chilled at excessively rapid rates may result in intracellular ice crystal formation due to

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inadequate water removal from the cells (Yusoff *et al.*, 2018). In other words, the results suggest that cooling at 3 to 7 cm for cryopreservation of *E. lanceolatus* sperm is optimal and avoids damage from intracellular ice formation. Freezing using the optimal cooling rates also caused the least cryoinjury by minimising the solution effects (Mazur, 2004).



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Figure 3: Sperm (A) PTM (%) and (B) post-thaw duration (s) of *E. lanceolatus* when cryopreserved at different heights (3, 5, 7, and 9 cm)

Note: Significant differences between treatments are noted with different subsets ($p < 0.05$)

When cooled at 9 cm, PTM was significantly lower compared to all other conditions. There was a significant decrease in PTM (%) compared to all other conditions ($p > 0.05$), suggesting that spermatozoa were over-dehydrated before being immersed in LN (Idris *et al.*, 2024). *E. lanceolatus* sperm may have been damaged by exposure to high-concentration solutions such as the cryodiluent, resulting in excessive dehydration when cooling was too slow.

Motility duration and velocity are closely related to the energy production and propulsion mechanisms within the sperm cell (Inaba & Shiba, 2018). Mitochondria in sperm cells produce ATP, which serves as the energy source and is important to maintain the sperm's movement (Inaba & Shiba, 2018). This indicates that TH did not differ from DMSO in ATP levels or mitochondrial damage in sperm cells during the freezing and thawing process. Thus, maintaining sperm motility, duration, and velocity after freezing is important for them to reach oocytes and fertilise eggs. In this regard, utilising cryopreserved sperm with the highest motility duration may increase the probability of fertilisation success.

Cryopreservation of fish sperm has also been reported to be species-specific (Mazur, 2004; Koh *et al.*, 2010). The underlying hypothesis is that different fish have sperm with different morphological and physiological characteristics, making some species more resistant to a wider range of cooling rates. Sperm of *E. fuscoguttatus* (Yusoff *et al.*, 2018) and *E. septemfasciatus* (Koh *et al.*, 2010) have both been reported to have a wide range of effective sperm cooling rates for cryopreservation. Our results suggest that *E. lanceolatus* is similar and has an effective wide range of cooling rates (3 to 7 cm) that can be applied in sperm cryopreservation. Yang *et al.* (2022) reported similar results using DMSO as a CPA, noting that optimal cooling for *E. lanceolatus* sperm cryopreservation occurred when samples were cooled at heights of 5 to 7 cm. This method of adjusting the height and floating of the polysterene frame on the surface of LN has previously been used successfully for sperm cryopreservation of *Pangasius nasutus* (Idris *et al.*, 2024, 2025), *Pangasius bocourti* (Yang *et al.*, 2023), *C. gariepinus* (Miskolczi *et al.*, 2005; Kovács *et al.*, 2010), *Pelteobagrus fulvidraco* (Pan *et al.*, 2008), *E. septemfasciatus* (Koh *et al.*, 2010), *E. fuscoguttatus* (Yusoff *et al.*, 2018), and *Anguilla japonica* (Koh *et al.*, 2017). Cryopreservation of sperm is commonly performed using programme freezers, which are expensive and highly technical equipment. Programme freezers allow specific, adjustable cooling rates, though they are costly, especially for small- or medium-scale breeders and farmers. The heat-based cooling protocol is therefore an alternative to using the programme freezer.

Conclusions

In conclusion, replacing DMSO with TH as a CPA for *E. lanceolatus* is possible, as all TH and THD concentrations used in this study were not significantly different from the control. The highest PTM values were obtained using 20% TH as a CPA, with the best cooling protocol being cooling the straws at 7 cm from the LN surface for one and a half minutes. The results suggest that grouper breeders can easily apply this simple protocol to promote sperm cryopreservation for small-scale farmers and breeders.

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Author Contributions Statement

Koh conceptualised the central research idea, provided the theoretical framework, and wrote and revised the article. Nurizzati, Eril, and Nurhanani conducted the research and collected data. Shamsul Bahri and Siti Ariza also helped improve the research design, assisted in supervising research progress, and provided critical comments, suggestions, and revisions to improve the manuscript.

Conflict of Interest Statement

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare the absence of conflicting interests with the funders.

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